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13. ABSTRACT *(Maximum 200 Words)*

We are interested in the molecular mechanisms involved in DNA replication arrest by the S phase DNA damage checkpoints. Using in vitro simian virus 40 DNA replication assays, we have found three factors that directly contribute to DNA damage-induced DNA replication arrest: Replication Protein A (RPA), DNA-dependent protein kinase (DNA-PK), and a yet to be identified replication inhibitor. Both DNA-PK and the unknown factor are functioned as trans-acting inhibitors. RPA is the major eukaryotic single-stranded DNA binding protein required for DNA replication, repair and recombination. Upon DNA damage, RPA is found hyperphosphorylated and redistributed within nuclei to form foci. These two events require the replication fork movement under certain DNA damaging agent treatments. However, there is also a replication-independent mechanism associated with the actions of some DNA damaging agents. We hypothesize that the requirement of on-going DNA replication to induce RPA modification and foci formation is dependent on types of DNA lesion. Our results also suggest that RPA could be used as a cellular DNA damage marker to study the actions of chemotherapeutic agents.

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Introduction:

When chromosomal DNA is damaged by natural causes or by chemotherapeutic agents, cellular checkpoint pathways are activated to preserve genome integrity. This leads to either cell cycle arrest and DNA repair, or programmed cell death. Loss of checkpoint regulation is highly associated with increased incidences of cancer. A better understanding of cellular responses to DNA damage is therefore important for cancer prevention and treatment. Our objective is to further study the molecular mechanisms that DNA replication is inhibited by the S phase DNA damage checkpoints.

It was previously demonstrated that extracts from cultured human cells treated with DNA-damaging anti-cancer drugs are deficient in their ability to support simian virus 40 (SV40) DNA replication *in vitro*. We found two mechanisms involved in this process: Induction of a trans-acting DNA replication inhibitor; and/or the loss function of replication protein A (RPA), which is a major eukaryotic single-stranded DNA binding protein that required for DNA replication, repair and recombination (McHugh et al., 1999; Liu et al., 2000). The goal of this project is to identify the DNA damage-induced trans-acting inhibitor; and to elucidate the mechanism that regulate RPA's function in DNA replication and repair.

My first year report is focused on the on going projects, and finished work is described in Key Research Accomplishments section.

Body

(1) Bizelesin treatment induces a trans-acting inhibitor that is not DNA-PK.

We found a trans-acting DNA replication inhibitor induced in bizelesin-treated cells (McHugh et al., 1999). As reported by Wang et al. (1999) that DNA-dependent protein kinase (DNA-PK) was induced in camptothecin-treated cells and functioned as a trans inhibitor for SV40 DNA replication in vitro. There is a possibility that bizelesin-induced trans-acting inhibitor is also DNA-PK. To answer this question, we examined the DNA-PK activity in extracts from cells treated with bizelesin and several other DNA damaging agents.

Adozelesin or low dose of C-1027 that does not induce trans-acting inhibitor (Liu et al., 2000; McHugh et al., 2001; Liu et al., submitted) did not induce DNA-PK activity (Fig. 1, A and B). High dose C-1027 that induced a trans-acting inhibitor (Liu et al., submitted) is able to induce DNA-PK activity in extracts of treated cells (Fig. 1B). These results are in agreed with the reported camptothecin studies (Wang et al., 1999). To our surprise, bizelesin treatment did not induce DNA-PK activity (Fig. 1C). This data suggested that bizelesin induced trans-acting inhibitor is not DNA-PK.

Since DNA-PK is presence in all the cell extracts (Fig. 1, striped bars) but mostly inactive (white bars), we suspect that DNA-PK activity in extracts from cells treated with 10 nM C-1027 might be induced by co-purified DNA fragments in the extracts which is generated by the DNA scission agent. Immobilized DNase I was used to treat extracts from mock- or C-1027-treated cells and then tested for DNA-PK activity. After DNase I treatment, the DNA-PK activity was abolished (Fig. 1D), suggested that the DNA-PK activity in extracts from cells treated with 10 nM C-1027 was induced by co-purified DNA fragments, an artifact for the in vitro system.

(2) RPA foci formation and RPA32 hyperphosphorylation are induced by treatment of DNA damaging agents.

As described in the original proposal, Shao et al. (1999) reported that RPA hyperphosphorylation induced by gamma radiation or camptothecin-treatment could be blocked by pre-treating the cells with DNA polymerase inhibitor aphidicolin. Last year, Rodrigo et al. (2000) had a similar observation in UV-irradiated cells. Their data suggested that, in DNA damaged cells, DNA replication fork movement is essential for subsequence RPA hyperphosphorylation. However, different results were observed when two other DNA damaging agents were applied to cells. RPA32 hyperphosphorylation induced by higher dose adozelesin was only partially blocked by aphidicolin pre-treatment (Liu et al., 2000). DNA scission agent C-1027 is able to induce high level of RPA32 hyperphosphorylation in treated cells with or without pre-treated with aphidicolin (McHugh et al., 2001). Our results suggest a DNA replication-independent mechanism for the induction of RPA32 hyperphosphorylation. We hypothesize that the requirement of DNA replication for the induction of RPA32 hyperphosphorylation is dependent on the type of DNA damage lesion.

Since RPA also form foci within nucleus when cellular DNA is damaged (Liu et al., submitted), it is monitored in DNA damaging agents treated cells by indirect immunofluorescent staining (Liu et al., submitted). Interestingly, the number of cells that have RPA foci varied among different drug treatments (Fig. 2, left panel). After treated with camptothecin-, bizelesin- or adozelesin, about 40% of the cells showed significant RPA foci (Fig.2, left panel) that disappeared if cells were pre-treated with aphidicolin (Fig. 2, right panel). This data implied the requirement of DNA replication fork movement to induce RPA foci. However, when high dose of adozelesin or 1 nM C-1027 were used, there are RPA foci formations that will not be affected by aphidicolin pre-treatment, suggesting a replication-independent mechanism is involved. These results are in parallel to RPA32 hyperphosphorylation studies (McHugh et al., 2001; Liu et al., 2000).

To further understand the relationship between DNA damage-induced RPA foci formation and DNA replication, we treated the cells with aphidicolin for 16 hr to arrest them in S phase. Cells were then treated with DNA damaging agents with or without first been released from aphidicolin block. As shown in Fig. 3, addition of adozelesin or C-1027 but not camptothecin or bizelesin induced RPA signal higher than mock treatment in aphidicolin arrested cells (left panel). Conversely, 1 hr after released from aphidicolin block, all DNA damaging agents tested induced RPA foci in more than 95% of the cells (Fig. 3, right panel). Our studies suggested that the dependency of DNA replication for RPA foci formation is also relied on different types of DNA damage lesion.

Key Research Accomplishments:

Task 1 has been finished and the results are very interesting. We further this project by using indirect immunostaining. Some of the results have been published or submitted for publication (Liu et al., 2000; McHugh et al., 2001; Liu et al., submitted), and the rest of them are presented in this report. This part of the project is going to be expanded in the future study.

Task 2 has been finished and the results have been submitted for publication (Liu et al., submitted).

Task 3 is 80% finished. Part of the results has been submitted for publication (Liu et al., submitted).

Task 5 is 70% finished. Phosphorylated RPA has been sent to Biopolymer Center at Roswell Park Cancer Institute for phosphorylation sites identification. RPA acetylation cannot be found in cells treated with DNA damaging agents and has been removed from this study.

Reportable Outcomes:

(1) Manuscript:

Mary M. McHugh, Xia Yin, Shu-Ru Kuo, Jen-Sing Liu, Thomas Melendy, and Terry A. Beerman (2001) The cellular response to DNA damage induced by the enediynes C-1027 and neocarzinostatin includes hyperphosphorylation and increased nuclear retention of Replication Protein A (RPA) and trans inhibition of DNA replication. Biochemistry 40:4792-4799.

Jen-Sing Liu*, Shu-Ru Kuo*, Xia Yin, Terry A. Beerman and Thomas Melendy· DNA damage results in the loss of replication protein A function through two distinct mechanisms. Submitted (*: Authors with equal contribution).

(2) Meeting Abstract:

Shu-Ru Kuo, Jen-Sing Liu, Xia Yin, Terry A. Beerman, and Thomas Melendy. Different DNA damaging agents trigger different DNA replication inhibition mechanisms.
"Eukaryotic DNA Replication Meeting", The Salk Institute, La Jolla, CA. Sept. 6-10, 2000.

(3) Grant Application:

"Mechanisms of DNA damage triggered S phase checkpoints"
PI: Thomas Melendy (Mentor of this project)
R01 CA89259-01 (NIH/NCI)
Jan, 2001-Dec, 2005

Conclusions:

(1) DNA-PK was suggested to be the trans-acting inhibitor induced by camptothecin treatment. Our data indicated that the DNA-PK activation might be an artifact of an in vitro system. However, activated DNA-PK does have the ability to inhibit SV40 DNA replication in vitro. The possible role of DNA-PK in DNA damage induced DNA replication arrest requires further studies. For bizelesin-induced trans-acting inhibitor, we will follow original proposed procedure to reveal its identity.

(2) Because of its pivotal role in both DNA replication and repair synthesis, RPA is considered a good target for cellular DNA damage checkpoints. For certain applications, RPA might be a better marker than others for measuring DNA damages. RPA is an abundant cellular protein that binds on single-stranded DNA as well as damaged double-stranded DNA (Lao et al., 2000) with or without DNA strand break. Most of other commonly used DNA damage markers require double-stranded DNA break ends for foci formation (Mirzoeva and Petrini, 2001). Therefore, RPA foci are a good indicator for any type of DNA damage inside cells.

Camptothecin has been used to treat cancer patients for decades. It is known that camptothecin induces S phase checkpoints in treated cells. Our data demonstrated the same finding using RPA as a marker. This S phase specific effect might be another good reason that camptothecin can be used to treat cancer efficiently. Conversely, C-1027 that causes damages in all treated cells will not be a good candidate for chemotherapy.

Although adozelesin and bizelesin damage DNA directly, RPA foci are found mostly in S phase cells. It suggests that (1) adozelesin or bizelesin associated DNA adducts might not be recognized by RPA directly, (2) they might induce the similar cellular response mechanism as camptothecin does, (3) since the removal of adozelesin, bizelesin induced DNA adducts is more difficult than of camptothecin induced adducts, it would require less drug to achieve a longer effect. Although it might also suggest a higher level of cell toxicity.

By further studying cellular DNA damaging responses, we would be able to understand how chemotherapeutic agents selectively kill tumor cells. Which would provide a good guideline for better therapeutic agents design with higher efficacy and lower side effect.

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McHugh, M. M., Yin, X., Kuo, S. R., Liu, J. S., Melendy, T., and Beerman, T. A. (2001) The cellular response to DNA damage induced by the enediynes C-1027 and neocarzinostatin includes hyperphosphorylation and increased nuclear retention of Replication Protein A (RPA) and trans inhibition of DNA replication. Biochemistry 40:4792-4799.

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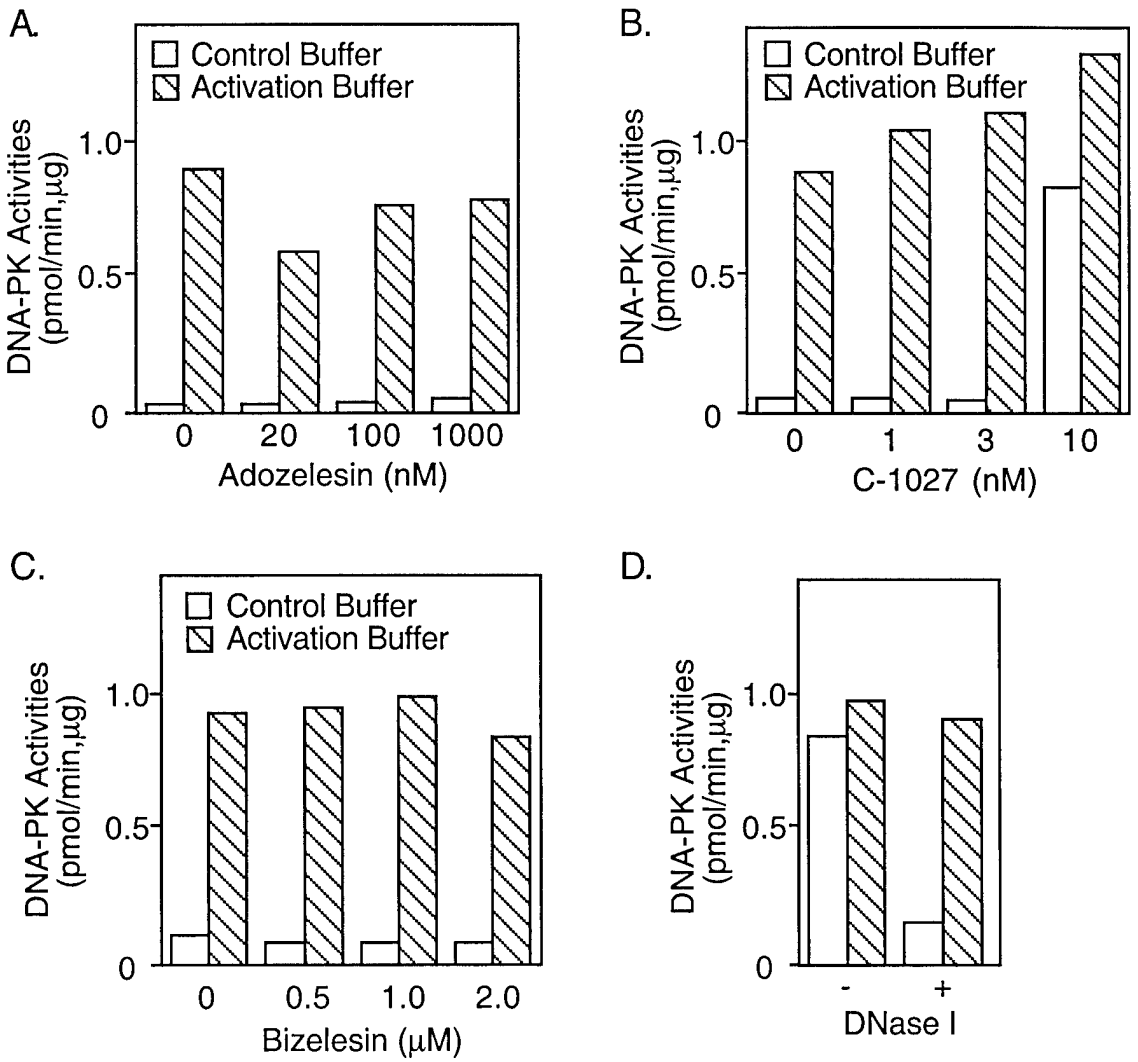
Figure 1.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Activation Buffer
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0
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Adozelesin (nM)
B.
Control Buffer
Activation Buffer
DNA-PK Activities (pmol/min,μg)
1.0
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C-1027 (nM)
C.
Control Buffer
Activation Buffer
DNA-PK Activities (pmol/min,μg)
1.0
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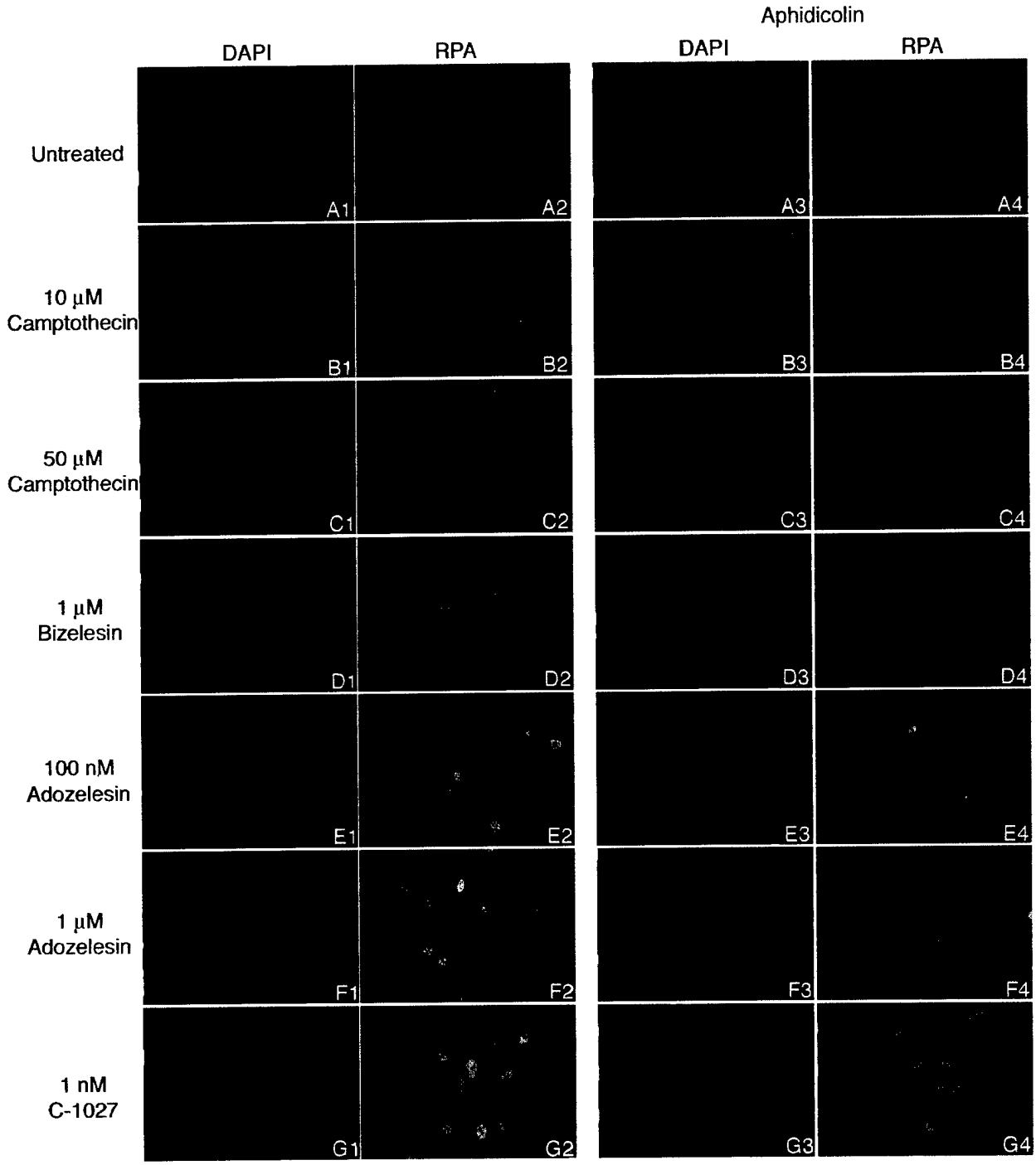
Figure 2.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DAPI
RPA
DAPI
RPA
Untreated
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A2
A3
A4
10 μM Camptothecin
B1
B2
B3
B4
50 μM Camptothecin
C1
C2
C3
C4
1 μM Bizelesin
D1
D2
D3
D4
100 nM Adozelesin
E1
E2
E3
E4
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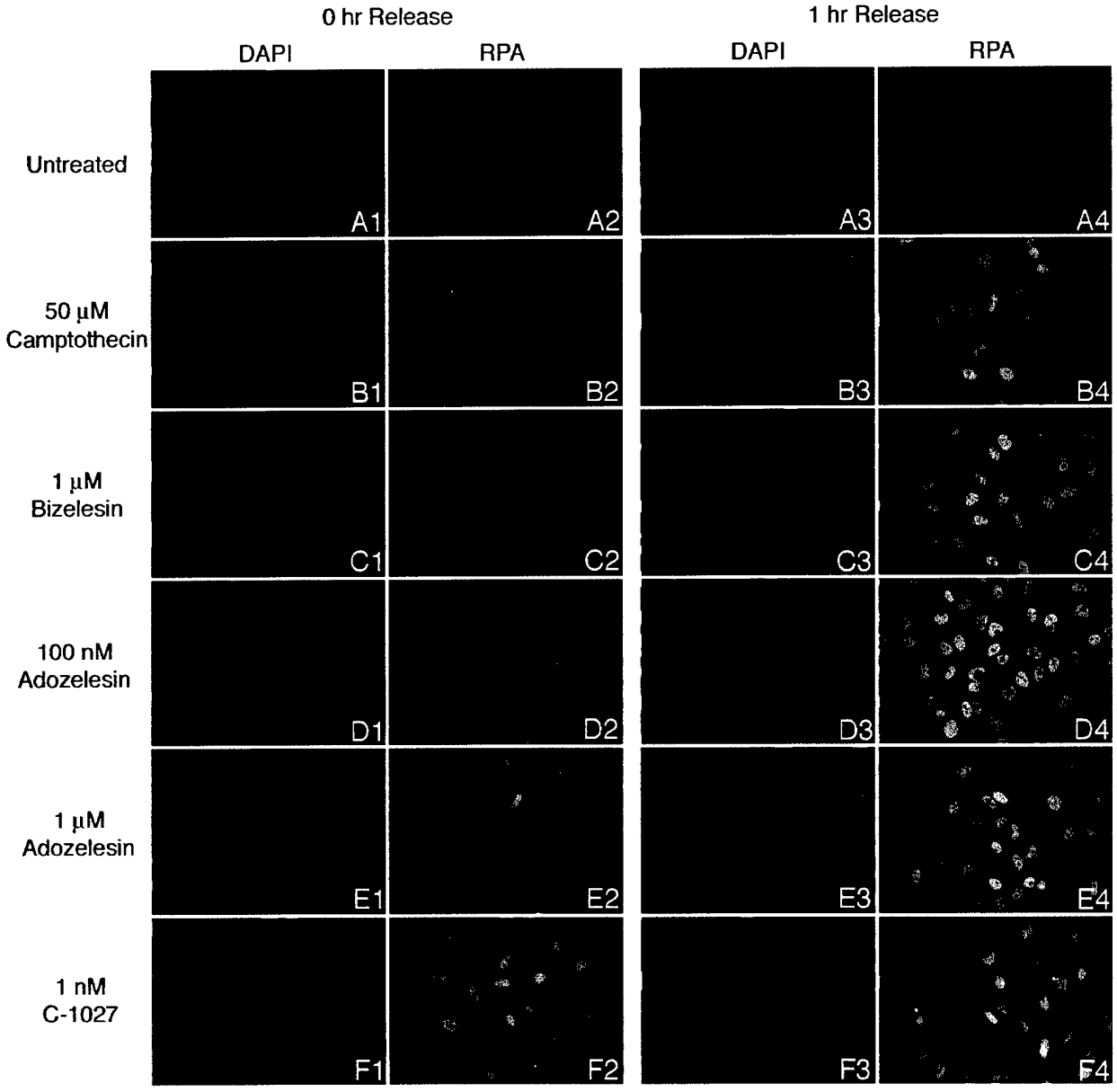
F1
F2
F3
F4
1 nM C-1027
G1
G2
G3
G4

Figure 3.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1 hr Release
DAPI
RPA
DAPI
RPA
Untreated
50 µM Camptothecin
1 µM Bizelesin
100 nM Adozelesin
1 µM Adozelesin
1 nM C-1027
A1
A2
A3
A4
B1
B2
B3
B4
C1
C2
C3
C4
D1
D2
D3
D4
E1
E2
E3
E4
F1
F2
F3
F4

Figure legends:

Figure 1. DNA-PK activities in cell extracts. DNA-PK activity in cell extracts was detected using a SignaTECT DNA-Dependent Protein Kinase System (Promega). Control buffer is 10 mM Tris-HCl pH 8.0; 1 mM EDTA; and Activation buffer consists of the same buffer with the addition of 100 ng/ml calf thymus DNA. Forty μg of total protein from extracts of cells treated with different drugs (A, adozelesin; B, C-1027; C, bizelesin; D, 10 nM C-1027) were tested for DNA-PK activity. Cell extracts in D had been treated with immobilized DNase I before kinase assays.

Figure 2. Indirect immunofluorescent staining of RPA. HeLa cells seeded on chamber-slides (Lab-Tek) were incubated with (right side panel) or without (left side panel) 2.5 μM of aphidicolin for an hour prior to addition of DNA damaging agents at a concentration indicated at left. After washed with PBS, pre-permeablized with 0.5% Triton X-100 in PBS, fixed with 3% paraformaldehyde in PBS and pre-blocked with 50% normal goat serum in PBS at R.T. for an hour, monoclonal antibody against RPA32 (3 mg/ml) was added as the primary antibody for another hour at 37°C. After wash with 0.5% Triton X-100 in PBS, fluorescein conjugated goat anti-mouse antibody (Vector Laboratory Inc.) and 2 μM DAPI were added at room temperature for 1 hr. The cells were then covered with mounting medium (Vector Laboratory Inc) after extensive wash, and examined under an Olympus BX40 microscope with SPOT-RT digital camera. Adobe Photoshop was used for image processing and printing. DAPI and RPA represented nucleus and RPA staining, respectively.

Figure 3. Indirect immunofluorescent staining of RPA in S phase cells. Twenty-five μM of aphidicolin were incubated with seeded HeLa cells for 16 hr before removed from complete medium for 0 hr (Left side panel) or 1 hr (right side panel). The following DNA damaging treatment and immunofluorescent staining were described in Figure 2. DAPI and RPA represented nucleus and RPA staining, respectively.

The Cellular Response to DNA Damage Induced by the Enediynes C-1027 and Neocarzinostatin Includes Hyperphosphorylation and Increased Nuclear Retention of Replication Protein A (RPA) and Trans Inhibition of DNA Replication†

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ABSTRACT: This study examined the cellular response to DNA damage induced by antitumor enediynes C-1027 and neocarzinostatin. Treatment of cells with either agent induced hyperphosphorylation of RPA32, the middle subunit of replication protein A, and increased nuclear retention of RPA. Nearly all of the RPA32 that was not readily extractable from the nucleus was hyperphosphorylated, compared to ≤50% of the soluble RPA. Enediyne concentrations that induced RPA32 hyperphosphorylation also decreased cell-free SV40 DNA replication competence in extracts of treated cells. This decrease did not result from damage to the DNA template, indicating trans-acting inhibition of DNA replication. Enediyne-induced RPA hyperphosphorylation was unaffected by the replication elongation inhibitor aphidicolin, suggesting that the cellular response to enediyne DNA damage was not dependent on elongation of replicating DNA. Neither recovery of replication competence nor reversal of RPA effects occurred when treated cells were further incubated in the absence of drug. C-1027 and neocarzinostatin doses that caused similar levels of DNA damage resulted in equivalent increases in RPA32 hyperphosphorylation and RPA nuclear retention and decreases in replication activity, suggesting a common response to enediyne-induced DNA damage. By contrast, DNA damage induced by C-1027 was at least 5-fold more cytotoxic than that induced by neocarzinostatin.

Enediyne drugs are highly cytotoxic protein antitumor antibiotics containing a reactive chromophore which binds in the minor groove of DNA (*1*, *2*). The cytotoxicity of enediyne drugs is directly correlated with chromophore-induced single- or double-strand DNA damage (*3*) which occurs via formation of a benzenoid diradical.

Despite their common dependence on a chromophore for DNA damage, enediynes exhibit striking differences. For example, the enediyne C-1027, which induces primarily double-stranded DNA damage (*4*, *5*), has significant cytotoxicity in the picomolar range (*6*, *7*). By contrast, primarily single-strand DNA damage is induced by the enediyne neocarzinostatin (*8*) which is 3 orders of magnitude less cytotoxic than C-1027 (*9*). C-1027 and neocarzinostatin also differ in the chemical structure of the activated chromophore (*10*) and in its nucleotide base preference (*11*). Despite these differences, within minutes of treatment with cytotoxic doses of either C-1027 or neocarzinostatin, both DNA synthesis and progression through the S phase are decreased.

Recently, we showed that C-1027 inhibited intracellular replication of SV40 DNA at drug levels that induced less than one lesion per viral DNA molecule (*12*). That the presence of a DNA lesion was not crucial for reduced replicative synthesis suggested a trans mechanism of C-1027 inhibition. Trans inhibition of replication can occur by induction of an inhibitor or by reduction in the amount or activity of essential replication factors. DNA damaging agents which induce an inhibitor of cell-free SV40 DNA replication include X irradiation (*13*), the topoisomerase I inhibitor camptothecin (*13*), and the DNA alkylator bizelesin (*14*). In contrast, reduction in the amount of an essential replication factor rather than induction of an inhibitor is observed after treatment with adozelesin, a DNA alkylator similar to bizelesin (*15*). To date, trans inhibitory effects on replication have not been reported for neocarzinostatin.

One essential replication factor that has been associated with the cellular trans response to DNA damage is the heterotrimeric replication protein A (RPA) (*16*). Damage to cellular DNA by X irradiation, camptothecin (*13*), and the DNA alkylator bizelesin (*15*) result in hyperphosphorylation of the middle subunit of RPA (RPA32), possibly via kinases such as DNA-PK and ATM (*17*). Addition of exogenous RPA failed to restore replication activity in cell-free studies of these agents, indicating that additional factors were involved in inhibiting replication activity. By contrast, decreased functional levels of RPA alone may account for

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the reduced replication activity observed when RPA hyperphosphorylation results from UV irradiation (*18*, *19*) or from treatment with the DNA alkylator adozelesin (*15*), since replication activity can be restored with exogenous RPA.

This study examined the relationship between DNA damage and inhibition of DNA replication induced by the enediynes C-1027 and neocarzinostatin. A cell-free SV40 DNA replication assay was used to examine whether enediynes inhibited replication competence in extracts of treated cells in a trans-acting manner. The role of phosphorylation and intracellular localization of RPA32 in enediyne-induced decreased replication competence was examined by Western blotting. The extent of enediyne-induced genomic DNA damage was quantitated to identify threshold levels of strand breaks necessary to reduce replication competence.

EXPERIMENTAL PROCEDURES

Chemicals. C-1027, a generous gift from Taiho Pharmaceutical Co., Ltd. (Saitama, Japan), was adjusted to 2 mg/mL in H_2O. Neocarzinostatin was obtained from Bristol-Myers Co. (Syracuse, NY) as a 2 mg/mL solution in 15 mM sodium acetate. Both stock solutions were stored at −20 °C. [2-^{14}C]Thymidine (55 mCi/mmol) was from Moravek Biochemicals, Inc. (Brea, CA). [α-^{32}P]dATP (3000 Ci/mmol) was from Amersham Pharmacia Biotech, Inc. (Piscataway, NJ). Aphidicolin from Sigma-Aldrich (St. Louis, MO) was diluted in ethanol and stored at −20 °C. All other chemicals were of reagent grade.

Cell Culture and Antibodies. Human 293 cells (adenovirus 5 DNA-transformed embryonic kidney cells) were maintained in monolayer culture in DMEM with 10% fetal bovine serum in a humidified atmosphere of 5% CO_2. Monoclonal antibodies against human RPA-70 and RPA-32 have been described previously (*20*).

Plasmids and Proteins. SV40 large T-antigen was purified from recombinant baculovirus-infected High-Five insect cells (Invitrogen) using immunoaffinity chromatography (*21*). SV40 origin-containing plasmid pSV011 has been described elsewhere (*22*).

Cytotoxicity Assays. Cells (3×10^5) were seeded in 60 mm plates. After 2 days, growth medium was replaced with 1.0 mL of medium containing C-1027 (0.1−100 pM) or neocarzinostatin (0.1−20 nM). After 2 h at 37 °C, cells were rinsed with warm phosphate-buffered saline (PBS), trypsinized, and resuspended in fresh medium. Cells were seeded at 500, 1000, or 3000 per 60 mm plate and incubated at 37 °C for 12 days. Plates were stained with 1% methylene blue and colonies counted. The number of colonies in plates containing drug-treated cells was compared to that in control plates containing nondrug-treated cells, and results were expressed as percent inhibition of colony formation.

Detection of Genomic DNA Damage. The procedure for assaying genomic DNA damage was described previously (*23*). Briefly, 293 cells (1×10^6/100 mm plate) were radiolabeled for 48 h with [2-^{14}C]thymidine (0.0125 μCi/mL) and then treated with C-1027 or neocarzinostatin for an additional 2 h. X-ray treatment of nondrug-treated cells was used as a positive control for DNA double-strand breaks. Drug-treated or irradiated cells were harvested and washed once in PBS. Cells were resuspended in PBS, and low gelling temperature agarose was added to a final concentration of 0.66%. Seven microliters of lysis buffer (1% Sarcosyl, 0.5 M EDTA, pH 8.0, 10 mg/mL proteinase K) was added to 21 μL of the cell−agarose mixture, and samples were incubated at 55 °C for 2 h. After incubation, samples were spun briefly and placed at 4 °C to allow a hardened agarose plug to form. Plugs were stored overnight in 0.5 mL of TE before loading on a 0.8% agarose gel. After pulsed-field gel electrophoresis for 90 h at 64 V with a 35 min pulse between field changes, gels were dried and exposed to a phosphorimager screen for detection of ^{14}C-radiolabeled DNA. The image was scanned, and lanes were quantitated using a Molecular Dynamics phosphorimager and ImageQuant software.

Cell-Free DNA Cleavage Assay. Drug-induced DNA breaks in the superhelical pSV011 plasmid DNA were measured using a topological form conversion assay. One hundred nanograms of DNA was incubated with C-1027 or neocarzinostatin in 10 mM Tris, pH 7.5, for 15 min at 37 °C. Dithiothreitol (0.5 mM) was included in the neocarzinostatin reaction buffer as an activating agent. After addition of 1% sodium dodecyl sulfate (SDS) to terminate the reaction, samples were electrophoresed on a 0.8% agarose gel. Gels were stained with ethidium bromide and analyzed using an Alpha Innotech Corp. (San Leandro, CA) Chemi-Imager.

Preparation of Cell Extracts for Cell-Free Replication Assays. Mid-log phase growing cells were seeded at 3.8×10^6/100 mm plate and grown overnight before treatment with C-1027 or neocarzinostatin. After 2 h at 37 °C, cells were harvested and extracts prepared as described previously (*14*). All steps were performed at 4 °C. Briefly, after one wash each in PBS and hypotonic buffer, cells were incubated in hypotonic buffer for 10 min and then lysed by seven passages through a 25-gauge needle. Samples were placed on ice for 30 min and centrifuged at 9000*g* for 10 min. The supernatants (soluble extracts) were removed, quick frozen in dry ice−propanol, and stored at −80° C until use. The pellets were washed twice in cold PBS, and 2 × SDS loading buffer (*24*) was added to each pellet to obtain the extraction-resistant nuclear fraction. Protein concentrations of the extracts were determined using the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Inc., Hercules, CA).

Cell-Free SV40 DNA Replication Assays. Cell-free replication assays were carried out as described previously (*14*, *15*) in a total volume of 10 μL. Thirty nanograms of SV40 origin containing plasmid pSV011, 500 ng of T-antigen, and 40 μg of soluble extract protein from control (no drug treatment) or enediyne-treated cells (or as indicated in the figure legends) were combined with replication assay buffer (4 mM ATP, 0.2 mM CTP, GTP, and UTP, 0.1 mM dCTP, dGTP, and dTTP, 0.025 mM dATP, 7.0 mM $MgCl_2$, 0.024 unit of creatine phosphokinase, 40 mM phosphocreatine, and 2 μCi of [α-^{32}P]dATP) and incubated at 37 °C for 60 min. Total incorporation of ^{32}P was determined by spotting samples on Whatman DE-81 paper filters and washing the filters. For analysis of DNA products, samples were separated by electrophoresis on a 0.8% (w/v) agarose gel in 1 × TAE.

Western Blots. Extracts prepared from control and treated cells as described above were electrophoresed on 10% SDS−PAGE and transferred to Hybond-P membrane [Amersham Pharmacia Biotech, Inc. (Piscataway, NJ)]. Membranes were

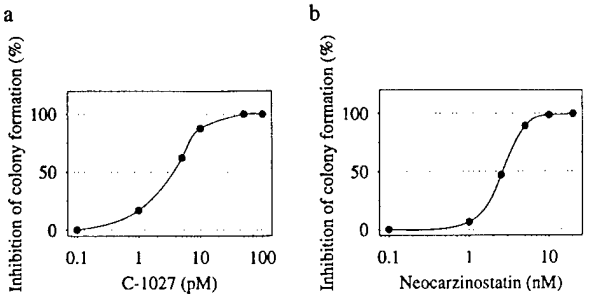


FIGURE 1: Cytotoxicity of C-1027 and neocarzinostatin. 293 cells were treated with the indicated concentrations of C-1027 (a) or neocarzinostatin (b). After 2 h, cells were washed in PBS, trypsinized, reseeded, and incubated at 37 °C for 12 days when colonies were counted. Data are the average of two independent experiments.

first probed with monoclonal antibodies against human RPA-70 and RPA-32 and then with peroxidase-conjugated anti-mouse IgG antibody. Blots were developed using the chemiluminescent ECL kit (Amersham Pharmacia Biotech).

RESULTS

Cytotoxicity of C-1027 and Neocarzinostatin in 293 Cells. This study investigates the cellular response to DNA damage induced by enediynes. Since response to DNA damage would likely be dependent on cytotoxic activity, it was necessary to quantitate C-1027- and neocarzinostatin-induced cytotoxicity in 293 cells (*7*, *25*, *26*). The cytotoxic effects of C-1027 and neocarzinostatin were assayed by colony formation. A 50% decrease in colony formation was observed 12 days after treatment with 3.6 pM C-1027 or 2.6 nM neocarzinostatin (see Figure 1). Thus, C-1027 was more than 700-fold more cytotoxic to 293 cells than was neocarzinostatin.

Genomic DNA Damage by C-1027 and Neocarzinostatin. To study the relationship between DNA damage and cellular responses that affect DNA replication, it was necessary to quantitate enediyne-induced intracellular DNA damage. Induction of genomic DNA double-strand damage was measured by pulsed-field gel electrophoresis.

Figure 2a is a representative phosphorimage of ^{14}C-radiolabeled DNA isolated from cells treated with increasing doses of X-ray, C-1027, or neocarzinostatin. The amount of DNA remaining in the well after pulsed-field gel electrophoresis was used to quantitate the extent of DNA double-strand damage (*23*). In the controls (lanes 1 and 7), the majority of radiolabel remained within the well. However, increased migration of ^{14}C-radiolabeled DNA into the gel was apparent with 0.5−5 nM C-1027 (lanes 8−10) and 0.5−2 μM neocarzinostatin (lanes 11−13). DNA from cells X-irradiated with 2−120 Gy (lanes 1−6) was used to compare enediynes with an agent that produces a predictable amount of DNA damage

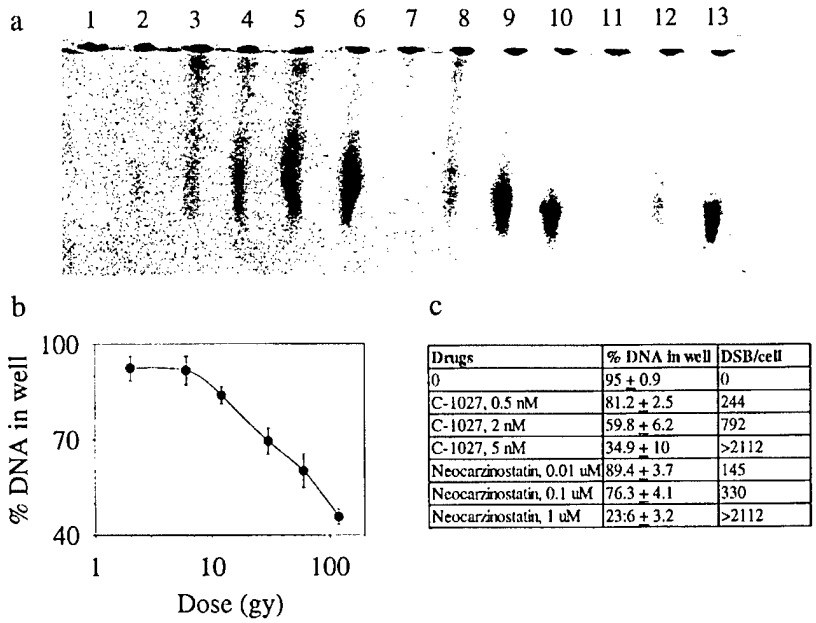
Figure 2b is a graphic representation of data from multiple pulse-field gels showing that the fraction of ^{14}C-radiolabeled DNA remaining in the well decreased as the X-ray dose increased. In control samples (no drug or X-ray treatment), the amount of DNA remaining in the well was typically 90−95% of the total ^{14}C signal. In samples subjected to X irradiation, a slight decrease in ^{14}C-radiolabeled DNA in the well was observed with 7 Gy and a 50% loss with 90 Gy.

The percent of DNA remaining in the well after C-1027 or neocarzinostatin treatment also decreased in a dose-dependent manner (Figure 2c). Double-strand DNA breaks (DSB) per cell were estimated by comparing the loss of DNA from the well in C-1027- or neocarzinostatin-treated samples with that observed after X irradiation. Similar levels of DNA damage were induced by 0.5 nM C-1027 and 0.1 μM neocarzinostatin (244 and 330 double-strand breaks per cell, respectively).

If double-strand DNA damage is assumed to be linear, the formation of double-strand breaks at cytotoxic doses of C-1027 and neocarzinostatin can be calculated from the data in Figures 1 and 2. At doses necessary to reduce colony formation by 50% (i.e., 3.6 pM C-1027 or 2.6 nM neocarzinostatin), the number of double-strand breaks induced per cell was 1.4−1.8 for C-1027 (calculated from DSB induced by 0.5 and 2 nM C-1027) and 8.6−37.7 for neocarzinostatin (calculated from DSB induced by 0.1 and 1.0 μM neocarzinostatin). Thus, at least five times more neocarzinostatin- than C-1027- induced double-strand breaks were necessary to cause cytotoxicity.

Reduced Cell-Free DNA Replication Competence in Soluble Extracts of Drug-Treated Cells. To examine whether damage to cellular DNA by enediynes leads to a loss of replication competence, the replication activity of soluble extracts of enediyne-treated 293 cells was determined using a cell-free SV40 DNA replication assay that has been described elsewhere (*27*, *28*). Superhelical pSV011 plasmid DNA which contains an SV40 origin fragment (*22*) was used as a template. Cells were treated for 2 h at 37 °C with C-1027 or neocarzinostatin, and soluble extracts were prepared. Figure 3a is a phosphorimage of a representative agarose gel after electrophoresis of cell-free DNA replication reactions. Twenty, thirty, or forty micrograms of total protein from each untreated control or C-1027-treated cell extract was used in replication assays. A slight decrease in total ^{32}P incorporation into newly replicated DNA was observed with as little as 0.5 nM C-1027 at all three protein levels. At 2.0 and 5.0 nM C-1027, the radioactive signal was reduced dramatically compared to the control (0) samples. Thus, C-1027 treatment of intact cells reduced in a dose-dependent manner the ability of cell extracts to replicate SV40 DNA.

A graphic representation of reduced replication activity in extracts from cells treated with C-1027 is shown in Figure 3b. When the amount of protein extract was increased from 20 to 40 μg, the replication activity increased in extracts from untreated (control) and 0.5 and 2.0 nM C-1027-treated cells, although drug-treated samples remained less active than control samples. A similar pattern of replication inhibition was observed after cells were treated with 0.01−1.0 μM neocarzinostatin (see Figure 3c). Treatment with 0.5 nM C-1027 reduced replication 20−30%, while treatment with 0.1 μM neocarzinostatin reduced activity by 31−39%. Thus, drug doses that induced similar levels of DNA damage [i.e., C-1027 (0.5−5.0 nM) or neocarzinostatin (0.1−1.0 μM) (see Figure 2)] caused equivalent decreases in cell-free replication competence. Replication activity observed in extracts of cells treated with the highest dose of C-1027 (5.0 nM) or neocarzinostatin (1.0 μM) increased only slightly when the amount of extract was doubled from 20 to 40 μg, indicating that inhibition at higher doses may not be reversed by



Drugs	% DNA in well	DSB/cell
0	95 ± 0.9	0
C-1027, 0.5 nM	81.2 ± 2.5	244
C-1027, 2 nM	59.8 ± 6.2	792
C-1027, 5 nM	34.9 ± 10	>2112
Neocarzinostatin, 0.01 uM	89.4 ± 3.7	145
Neocarzinostatin, 0.1 uM	76.3 ± 4.1	330
Neocarzinostatin, 1 uM	23:6 ± 3.2	>2112

FIGURE 2: DNA damage in 293 cells induced by C-1027 and neocarzinostatin detected by pulsed-field gel electrophoresis (PFGE). (a) Representative phosphorimage showing increased migration of DNA into the gel when cells were treated with DNA damaging agents. Lanes 1 and 7: DNA from untreated cells. Lanes 2−6 contain DNA from cells irradiated prior to proteinase K digestion with 2 (1), 6 (2), 12 (3), 30 (4), 60 (5), or 120 (6) gray (Gy). Lanes 8−10: DNA from cells treated with 0.5 (8), 2 (9), or 5 (10) nM C-1027. Lanes 11−13: DNA from cells treated with 0.01 (11), 0.1 (12), or 1.0 (13) μM neocarzinostatin. (b) Graphic representation of the amount of DNA remaining in the well after PAGE of X-irradiated cells. (c) Summary of DNA damage induced by C-1027 and neocarzinostatin. Rad equivalent damage, estimated by comparing the loss of DNA from the well in C-1027-treated samples with that observed after X irradiation, was converted to double-strand breaks (DSB). Using a molecular mass of 320 Da per nucleotide, DSB per cell (6.6×10^9 nucleotides) were calculated by multiplying the rad equivalent damage by 8.3×10^{-14} DSB Da^{-1} $rad^{-1} \times 320$ Da $\times 6.6 \times 10^9$ nucleotides.

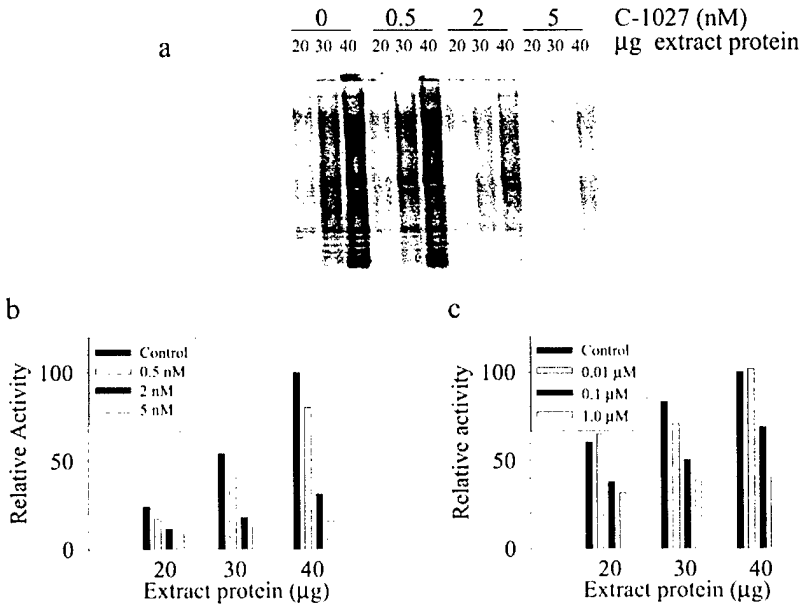


FIGURE 3: Cell-free SV40 DNA replication using soluble extracts from drug-treated cells. 293 cells were treated with the indicated concentrations of drugs for 2 h, and soluble extracts were prepared. A total of 20−40 μg of extract protein was used in reactions. (a) Phosphorimage of an agarose gel after electrophoresis of DNA replication reactions containing soluble extracts from cells treated with the indicated concentrations of C-1027. (b, c) Graphic representation of SV40 replication activity in soluble extracts from C-1027- or neocarzinostatin-treated cells, respectively.

supplementation with additional cellular replication proteins (i.e., additional extract).

Reduced Cell-Free Replication Competence Was Not Due to Damage to the DNA Template. To exclude the possibility that reduced cell-free replication competence was due to direct drug-induced damage to the DNA template, a cell-free DNA cleavage assay was carried out. Cleavage was identified as conversion of pSV011 supercoiled (form I) to nicked circular (form II) or linear (form III) DNA. Figure 4a shows the electrophoretic migration of pSV011 topological forms after incubation in the absence (control) or presence of 1−500 nM C-1027. Only high concentrations of C-1027

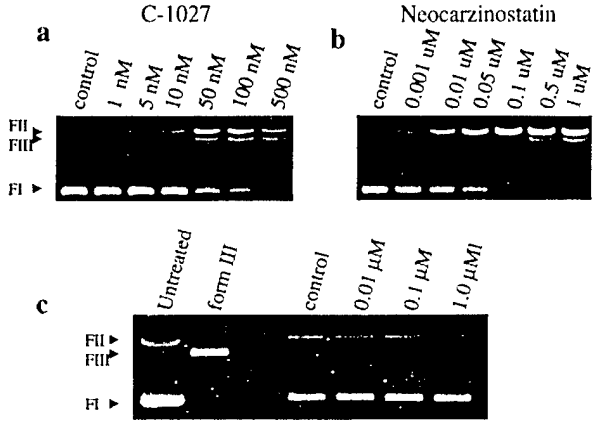


FIGURE 4: Cleavage of purified SV40 DNA by C-1027 or neocarzinostatin. pSV011 DNA was treated with the indicated concentrations of C-1027 (a) or neocarzinostatin (b) for 15 min at 37 °C and electrophoresed on a 1% agarose gel in 1 μg/mL ethidium bromide. A decrease in supercoiled form I (FI) and an increase in nicked circular form II (FII) or linear form III (FIII) were indicative of DNA damage. (c) A total of 40 μg of soluble extract protein from neocarzinostatin-treated cells was incubated with pSV011 DNA for 1 h at 37 °C. After proteinase K digestion and phenol/chloroform extraction, samples were electrophoresed as above. Lane 1 contained FI and FII and lane 2 contained FIII pSV011. Lanes 3−6 contain DNA incubated with soluble extracts from cells treated with the indicated concentrations of neocarzinostatin.

(10−500 nM) induced a progressive increase in form III and decrease in form I, indicative of DNA double-strand damage. However, after treatment with 1 and 5 nM C-1027, 85−90% of the DNA migrated as intact superhelical form I, similar to control samples. Since this was the dose range used for treating intact cells, damage to the template probably does not contribute to C-1027 inhibition of cell-free SV40 DNA replication.

However, with neocarzinostatin, damage was observed with as little as 0.01 μM and complete conversion of form I to form II or form III was noted with 1 μM neocarzinostatin treatment (see Figure 4b). While this is the neocarzinostatin range used to treat intact cells, it is likely that the actual drug levels present in the extract are lower, since cells were washed with PBS after neocarzinostatin treatment and the drug levels diluted during extract preparation. To determine whether any neocarzinostatin remained in the extract that could damage the DNA template, pSV011 was incubated with cell extract for 1 h at 37 °C. Figure 4c shows pSV011 DNA after incubation with soluble extracts from cells treated with 0, 0.01, 0.1, or 1.0 μM neocarzinostatin. No damage to DNA (i.e., no loss of form I or increase in forms II or III) was observed. Thus, direct damage to the DNA template due to residual amounts of neocarzinostatin in the cell extract probably does not contribute to the inhibition of cell-free replication.

Lack of a Trans-Acting Replication Inhibitor in C-1027- or Neocarzinostatin-Treated Extracts. C-1027 was reported earlier to inhibit DNA replication in trans (*12*, *29*), and a trans-acting inhibitor has been described in extracts from X-irradiated cells and in camptothecin- and bizelesin-treated cells (*13*, *14*). Mixing experiments were designed to determine whether enediynes also induce a trans-acting inhibitor. To 40 μg of control extract was added additional extract (10, 20, or 30 μg) from control or drug-treated cells, and cell-free SV40 DNA replication activity was measured. Figure 5a shows the effect of extracts from 2 or 5 nM C-1027-treated cells on the replication activity of control cell extracts. In the absence of additional extract the relative activity of 40 μg of control extract was 100%. Addition of 10−30 μg of control extract to the original 40 μg caused a progressive increase in replication activity. An increase in activity was also noted when extracts from cells treated with 2 or 5 nM C-1027 were added, although the increase was less pronounced. Since the effect of extracts from C-1027-treated cells on replication by control cell extract was additive, reduced replication activity in the drug-treated extracts was not due to the presence of an inhibitor.

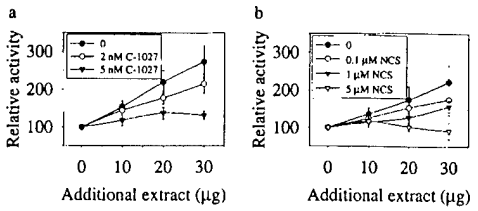


FIGURE 5: Mixing of enediyne-treated soluble extracts with control cell extracts does not indicate the presence of a trans-acting DNA replication inhibitor. Ten, twenty, or thirty micrograms of additional soluble extracts from mock- or enediyne-treated cells was added to 40 μg of mock-treated cell extract in cell-free SV40 DNA replication reactions. DNA products were isolated and analyzed as in Figure 3. DNA synthesis levels for each reaction are plotted as a percent of the activity present in the control (40 μg) reaction. Control soluble extract was mixed with the indicated amount of soluble extract from (a) C-1027-treated or (b) neocarzinostatin-(NCS-) treated cells. Data are the average of three experiments ± SEM.

Similar effects were observed with extracts from cells treated with 0.1 or 1.0 μM neocarzinostatin (Figure 5b). However, adding 20−30 μg of extract from cells treated with 5 μM neocarzinostatin to replication reactions did not increase activity over that in the presence of 40 μg of control extract alone. Although no additive effect was observed, the presence of an inhibitor of replication also was not indicated, since a reduction in activity below control levels did not occur.

Increased Retention in the Nucleus of RPA and Altered Electrophoretic Migration of RPA32 in Response to DNA Damage by C-1027 and Neocarzinostatin. Recent reports have described changes in RPA in response to DNA damage induced by various agents and suggest that RPA modification may be associated with a loss of replication competence similar to that seen in Figure 3 (for review, see ref *17*). To examine whether RPA was altered by enediyne treatment, the levels of RPA70 and RPA32 in the soluble and in the extraction-resistant nuclear fraction were determined by Western blotting. Figure 6a shows the distribution of RPA32 and RPA70 in cells treated with 0.5−5 nM C-1027 or 0.01−1 μM neocarzinostatin. With increasing C-1027 or neocarzinostatin treatment, the amount of RPA in the soluble extract decreased in a dose-dependent manner. The decrease in soluble RPA was accompanied by an increase in both RPA70 and RPA32 in the extraction-resistant nuclear fraction (see lower panel, Figure 6a). In addition, at higher drug doses (i.e., 2 and 5 nM C-1027 and 0.1 and 1 μM neocarzinostatin) a slower migrating band recognized by the RPA32 antibody was observed. At the higher drug doses, the slower migrating band accounted for about 50% of soluble and nearly 100%

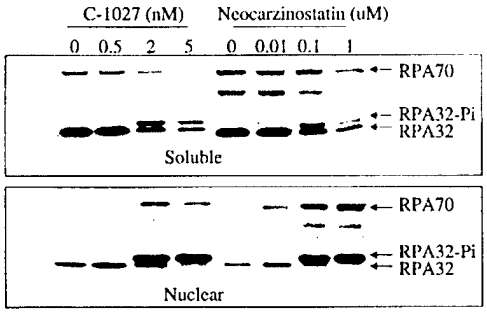


FIGURE 6: Changes in RPA induced by C-1027 or neocarzinostatin. Soluble extracts and extraction-resistant nuclear fractions prepared from cells treated with the indicated concentrations of C-1027 or neocarzinostatin were electrophoresed on 10% PAGE, subjected to Western blotting, and probed with monoclonal anti-RPA32 and RPA70 antibodies. Shown are phosphorimages of representative Western blots. A single RPA70 band and two RPA32 bands (RPA32-P_i and RPA32) are indicated by arrows.

FIGURE 7: The enediyne-induced RPA32-P_i band is hyperphosphorylated RPA32. To prove that the slower migrating RPA32 band (RPA32-P_i) in Figure 6 was hyperphosphorylated RPA32, samples were treated with alkaline phosphatase as described in Experimental Procedures. A total of 10 μg of proteins from extracts of control (lanes 1 and 2) or 3 nM C-1027- (lanes 3 and 4) treated cells was incubated with (lanes 2 and 4) or without (lanes 1 and 3) 7 units of calf intestine alkaline phosphatase (CIAP).

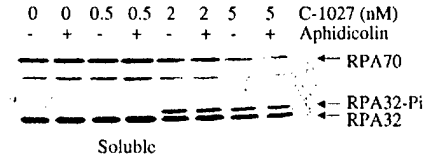


FIGURE 8: Aphidicolin does not prevent enediyne-induced RPA alterations. Cells were incubated for 15 min with 5 μM aphidicolin prior to addition of C-1027 at the indicated concentrations. Preparation of extracts and Western blot analysis were performed as described in Figure 6.

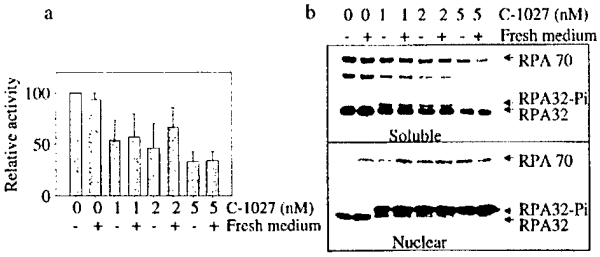


FIGURE 9: Replication inhibition and RPA hyperphosphorylation are not reversible by incubation in enediyne-free medium. Cells were treated with 1, 2, or 5 nM C-1027. After 2 h, half of the cells were harvested. The remaining cells were washed with fresh medium and further incubated in fresh medium for 4 h before harvesting, and extracts were prepared. (a) Cell-free SV40 DNA replication activity of 30 μg of soluble extract protein. Data are the average of four to six independent experiments and are expressed as the percent activity in control cell extracts. (b) Equal amounts of soluble and extraction-resistant nuclear fractions from a representative experiment were electrophoresed on 10% SDS–PAGE. RPA was detected by Western blotting using anti-RPA70 and anti-RPA32 monoclonal antibodies.

of extraction-resistant nuclear RPA32 signal. Thus, treatment with either enediyne caused increased RPA retention in the nucleus and altered RPA32 electrophoretic migration.

The Enediyne-Induced RPA32 Band with Decreased Electrophoretic Migration Is Hyperphosphorylated RPA32. DNA damage induces hyperphosphorylation of RPA32 which exhibits reduced electrophoretic migration compared to underphosphorylated RPA32 (*15*). To confirm that the slower migrating band seen after treatment with C-1027 or neocarzinostatin was hyperphosphorylated RPA, soluble extracts were treated with alkaline phosphatase as described in Experimental Procedures. Figure 7 is a Western blot showing the effect of alkaline phosphatase treatment on extracts from untreated (mock) and 3 nM C-1027-treated cells. Alkaline phosphatase had no effect on the faster migrating RPA32 band from mock- or C-1027-treated cells (lanes 1–4). However, phosphatase treatment caused the disappearance of the slower migrating RPA32 band (lane 3 compared to lane 4), indicating that hyperphosphorylation of RPA32 was responsible for the reduced band migration. Thus, the slower migrating RPA32 band in Figure 6 was defined as RPA32-P_i.

Enediyne-Induced RPA32 Hyperphosphorylation Is Not Dependent upon Continuing DNA Replication. Hyperphosphorylation of RPA induced by DNA damaging agents such as adozelesin, bizelesin, X irradiation, camptothecin, and UV is dependent on continuing DNA replication (*15*, *30*, *31*). Whether enediyne-induced hyperphosphorylation was similarly dependent was assayed by incubating cells with the well-known DNA fork elongation inhibitor aphidicolin (*32*–*34*). Pretreatment of cells with 5 μM aphidicolin prevented the appearance of a hyperphosphorylated RPA32 band in Western blots of extracts from 293 cells treated with 1 μM camptothecin (data not shown). However, aphidicolin had no effect on RPA32 hyperphosphorylation induced by C-1027 (see Figure 8) or neocarzinostatin (data not shown). Thus, control of RPA hyperphosphorylation induced by either enediyne differs from that of other DNA damaging agents.

Reversal of Enediyne-Induced Replication Inhibition and RPA Alterations Was Not Observed. The ability of C-1027-treated cells to recover their replication competence and reverse drug-induced changes in RPA was determined as described in Experimental Procedures. Cells were treated for 2 h with C-1027 and either harvested immediately or further incubated in fresh medium for 4 h. Figure 9a shows the relative replication activity in extracts from control cells or cells treated with 1–5 nM C-1027. Replication competence was unchanged whether cells were harvested immediately or further incubated to allow for reversal of drug-induced effects. The amount of enediyne-induced hyperphosphorylated RPA associated with the extraction-resistant nuclear fraction was the same with or without 4 h postdrug incubation in fresh medium (see Figure 9b). A similar lack of reversibility was observed with neocarzinostatin, even when the incubation time in fresh medium was increased to 16 h (data not shown). Thus, enediyne-induced effects on replication and RPA hyperphosphorylation are essentially irreversible.

DISCUSSION

A decrease in cell-free replication competence was observed in soluble extracts of cells treated with C-1027 or

neocarzinostatin doses that induced threshold levels of intracellular DNA damage. This decrease was not dependent on damage to the DNA template in the cell-free replication reaction, indicating for the first time that neocarzinostatin, like C-1027 (*12*), can inhibit DNA replication in trans.

Enediyne concentrations that decreased replication activity also caused hyperphosphorylation of RPA32, the middle subunit of RPA. Changes in RPA phosphorylation after DNA damage induced by a wide variety of agents [e.g., X irradiation (*35*, *36*), UV irradiation (*19*, *31*), and the topoisomerase inhibitor camptothecin (*30*, *37*), as well as the DNA alkylating agents bizelesin and adozelesin (*14*, *15*)] have been reported elsewhere. As with X irradiation and camptothecin, nearly 50% of soluble RPA32 becomes hyperphosphorylated after treatment with enediynes. The present study also showed that enediynes effected a large change in the intracellular distribution of RPA leading to association of the majority of cellular RPA with the extraction-resistant nuclear fraction. Nearly 100% of the RPA32 tightly associated with the nuclear fraction was hyperphosphorylated. This is the first report of a DNA damaging agent causing increased tight binding to the nuclear fraction of RPA70 and underphosphorylated and hyperphosphorylated RPA32. Thus, the RPA phosphorylation response to enediyne treatment may differ from that reported for other DNA damaging agents.

RPA32 is phosphorylated during the S phase of a normal cell cycle (*20*, *38*) as well as in response to DNA damage. Hyperphosphorylated RPA reportedly localizes to DNA single-strand regions (*39*), such as sites for DNA replication and repair. Enediyne-induced nuclear extraction-resistant hyperphosphorylated RPA32 may be localized at repair sites, limiting the ability of RPA to assemble at replication foci and to function in replication. Thus, in intact cells, enediyne-induced DNA damage may inhibit replication by altering RPA subunits to prevent their functional association with replicating foci.

In addition to increased RPA32 hyperphosphorylation, reduced levels of RPA70 and RPA32 were observed in the soluble extract from cells treated with either C-1027 or neocarzinostatin (see Figure 6). This loss, accompanied by decreased cell-free SV40 DNA replication activity, was observed after treatment with 0.5−5.0 nM C-1027 or 0.1−1.0 μM neocarzinostatin. Replication activity can be restored to soluble extracts from cells treated with 1 or 3 nM C-1027 by adding back RPA (manuscript in preparation). Thus, at the C-1027 concentrations used in the present study, reduced amounts of functional RPA, rather than induction of a replication inhibitor, likely account for the decreased replication competence of the soluble cell extract. This response to DNA strand damage is similar to that observed with the DNA alkylator adozelesin,which also decreased cell-free SV40 DNA replication competence in an RPA-associated manner (*15*). While no decrease in RPA levels in the soluble extract was noted after adozelesin treatment, RPA hyperphosphorylation was observed, and replication activity could be restored to the soluble extract by addition of RPA. Thus, a similar mechanism for inhibition of DNA replication resulting from decreased functional levels of RPA was observed with agents that differed dramatically in the type of DNA damage induced.

Decreased replication competence and reduced levels of RPA also have been described in soluble extracts from X-ray- and camptothecin-treated cells (*13*). However, replication activity inhibited by X-ray, by camptothecin, or by bizelesin, the bifunctional analogue of adozelesin (*14*), could not be restored by addition of exogenous RPA. Rather, the dominant replication effect of these agents was induction of an inhibitor, possibly DNA-PK. Thus, while the cellular response to treatment with a variety of DNA damaging agents may include a reduction in RPA levels, not all decreases in replication activity can be restored by adding back RPA, and the precise mechanism for replication inhibition may differ with specific DNA damaging agents.

Another example of the differing effects on replication caused by DNA reactive agents is the response to DNA damage after treatment with the DNA elongation inhibitor, aphidicolin. In contrast to RPA hyperphosphorylation induced by camptothecin, or the DNA alkylators adozelesin or bizelesin, that induced by C-1027 or neocarzinostatin is unaffected by aphidicolin pretreatment (Figure 8 and data not shown). Thus, cells can detect enediyne-induced DNA damage even in the absence of replication fork movement.

Recent studies on cellular recognition of DNA damage may partially explain this finding. An early step in DNA damage recognition reportedly involves thermodynamic probing of the duplex to identify agents which either stabilize (e.g., the alkylator CC-1065) or destabilize the DNA helix (*40*). Adozelesin and bizelesin, both analogues of CC-1065 (*60*,*61*) form bulky single- or double-strand adducts, but not DNA breaks (*41*), at the DNA−drug binding site, presumably stabilizing the DNA helix. Readily reversible protein-associated single-strand breaks are induced by camptothecin treatment (*42*), and these breaks also should have limited effects on helix destabilization. Thus, collision of the moving replication fork with these lesions might be required to enable DNA damage recognition and subsequent phosphorylation of RPA. By contrast, C-1027 and neocarzinostatin induce strand breaks which result from site-specific free radical attack on sugar moieties in both strands of DNA (*26*). These lesions directly reduce DNA superhelicity (*5*, *43*), effectively altering the tertiary structure of the DNA helix. Recently, RPA, which participates in multiple steps of nucleotide excision repair (NER) including the damage recognition step, was found to have increased affinity for sites where the double helix had been disrupted (*44*). RPA recognition of enediyne-induced effects on helical structure provides a possible pathway for recognition of enediyne-induced DNA damage which is independent of continuing DNA replication.

The minimum number of DNA double-strand breaks per cell necessary to decrease replication competence and induce RPA32 hyperphosphorylation and increased levels of nuclear extraction-resistant RPA was similar for C-1027 and neocarzinostatin. This suggested that a threshold level of damage was necessary to inhibit replication regardless of the enediyne examined. By contrast, when the cytotoxicity of the DNA lesions was assayed, differences between C-1027 and neocarzinostatin were observed. Extrapolation of the double-strand break damage shown in Figure 2 to drug levels, which caused a 50% reduction in colony formation, revealed a double-strand break frequency per cell of 1.3−1.6 for C-1027 compared to 7.8−34 for neocarzinostatin. Thus, damage

induced by C-1027 was at least 5-fold more lethal than that induced by neocarzinostatin.

One explanation for this difference may be the repairability of C-1027- and neocarzinostain-induced DNA lesions. C-1027 affects primarily double-strand damage, while double-strand breaks induced by neocarzinostatin probably result from single-strand breaks closely spaced on opposite strands (*45*). Single-strand DNA breaks induced by neocarzinostatin are readily repaired, and such repair should lead over time to reduced numbers of double- as well as single-strand breaks. Studies with repair enzymes also indicate a difference in repairability of lesions induced by differing enediynes. Other workers have shown that human apurinic/apyrimidinic endonuclease 1 (Ape 1) and *Escherichia coli* exonuclease III can partially repair DNA strand breaks produced by neocarzinostatin but not those induced by the enediyne calicheamicin (*46*). C-1027 contains an enediyne chromophore of the esperamicin/calicheamicin type, and it is possible that C-1027-induced lesions are not repaired as readily as those induced by neocarzinostatin. However, it is unlikely that differences in lesion repairability influence replication effects since RPA hyperphosphorylation induced by either enediyne was irreversible (see Figure 8), and the replication response to both drugs was very similar at concentrations affecting equivalent amounts of DNA damage.

In summary, both C-1027 and neocarzinostatin produced similar decreases in cell-free SV40 DNA replication competence with equivalent amounts of DNA double-strand damage. Future studies will examine enediyne-induced alterations in other replication and proliferation factors (e.g., the cyclin/cdk and Rb families) that may contribute to the DNA damage response pathways leading to replication inhibition. Whether a threshold of genomic damage similar to that induced by C-1027 and neocarzinostatin is crucial for triggering cellular response by other types of DNA damaging agents will be investigated.

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DNA DAMAGE RESULTS IN THE LOSS OF REPLICATION PROTEIN A FUNCTION THROUGH TWO DISTINCT MECHANISMS*

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Running Title: RPA in DNA damage-triggered replication arrest

Abstract

Within the past few years several studies have shown that treatment of cells with various DNA damaging agents results in a decrease in the ability of extracts from these cells to support *in vitro* DNA replication. In several cases this decrease in DNA replication activity has been attributed to an apparent insufficiency in Replication Protein A (RPA) function. It is shown here that DNA damage can result in loss of RPA function through two distinct mechanisms. One is through a decrease in available RPA. Although there is no change in the overall levels of cellular RPA, following certain types of DNA damage RPA becomes rapidly sequestered into intra-nuclear foci, resulting in a decrease in RPA available for DNA replication. The second mechanism is through inactivation of RPA for DNA replication function. Following DNA damage, RPA purified from treated cells shows a dramatically decreased ability to support SV40 DNA replication *in vitro* as compared to RPA from untreated cells. However, RPA from treated cells shows normal levels of single-strand DNA binding. Intriguingly, these finding suggest that the 'inactivation' of RPA might be specific for DNA replication, yet leave RPA functional for DNA repair.

Introduction

DNA damage in living cells results in the inhibition of DNA synthesis. Checkpoints have evolved to prevent synthesis or partitioning of the DNA genome when DNA damage is detected. Presumably these pathways have evolved because of the low copy number and essential nature of the genetic material, and the need to repair damaged genomic DNA prior to either DNA synthesis or cell division. While a great deal has been learned about how the G1 and G2/M checkpoints act to halt the cell cycle, substantially less is known about the S phase DNA damage checkpoint pathways. Most studies of S phase checkpoints have used genetic approaches in yeast systems (for reviews see 1-3). Mutants that compromise S phase checkpoint function include a variety of proteins such as: novel DNA interacting proteins, various kinases, and proteins known to be involved in DNA replication. One such yeast mutant is rfa1 M2, a mutation that maps to the largest of the three subunits of Replication Protein A (RPA)[1] (4). It is not altogether surprising that RPA should be one target of the DNA damage response pathways, since RPA is the major eukaryotic single-strand DNA (ssDNA) binding protein required for DNA replication, DNA repair, and recombination (for reviews see 5, 6).

Direct biochemical studies have also been used to examine S phase DNA damage checkpoint responses. Some studies have made use of the *in vitro* simian virus 40 (SV40) DNA replication system (7). Several of these reports have demonstrated that this *in vitro* system appears to accurately reflect the *in vivo* DNA damage response. Specifically, levels of inhibition of *in vitro* SV40 DNA replication using extracts from DNA damage-treated uninfected cells are similar to levels of inhibition of both SV40 and cellular DNA synthesis in infected and uninfected cells, respectively. Further, levels

of replication inhibition seen with various DNA damaging treatments have shown the same kinetics and dose response *in vivo* and *in vitro* (8-12). The mechanisms of inhibition of DNA replication triggered by different DNA damaging treatments fall into two classes. Several agents induce the presence of trans-acting DNA replication inhibitors (8, 13-15). Treatment of cells with other DNA damaging agents (specifically UV irradiation, the DNA alkylator adozelesin, and shown herein, the double-strand DNA (dsDNA) scission agent, C-1027) result in an apparent loss of RPA function. This is demonstrated by the ability of exogenously added RPA to rescue DNA replication activity in extracts from treated cells (16-18). In addition, the middle subunit of RPA (RPA32) has been shown to be hyper-phosphorylated in response to treatment of cells with various DNA damaging agents (5, 16, 19, 20-24). This hyper-phosphorylation appears to be predominantly due to DNA-dependent protein kinase (DNA-PK). However, *in vitro* phosphorylation of RPA by DNA-PK, either alone or in combination with cyclin A/cdk2, appears to have no effect on RPA function. These biochemical studies further support a major role for RPA in S phase checkpoint responses, although the mechanisms of RPA's involvement remain unknown.

For our studies on the role of RPA in S phase checkpoint mechanisms, we have chosen to use DNA damaging anti-cancer drugs. We have done so for two reasons. One, unlike more commonly used DNA damaging agents (such as γ and UV radiation), the drugs used each creates very specific types of lesion, rather than a wide variety of lesions. Two, the use of soluble drugs allows us to treat large cultures of cells with equivalent, highly reproducible levels of DNA damage. In this study we have focused on one DNA damaging agent, C-1027, to further elucidate the mechanisms of DNA-damage triggered S phase checkpoints. C-1027 is a DNA-specific strand scission agent that generates both ssDNA and dsDNA breaks, preferentially creating double strand

cleavages (25, 26). Like several of the agents discussed above, C-1027 has been shown to trigger the S phase checkpoint response and to arrest SV40 DNA replication at the same levels of drug that result in the inhibition of thymidine incorporation by the cellular replication machinery (10, 12). We have shown previously that extracts from cells treated with C-1027 show decreased levels of DNA replication *in vitro* (26). This current study investigates the mechanisms of this inhibition of DNA replication, focusing primarily on the mechanisms that lead to a loss of RPA function. It is shown herein that treatment of cells with low levels of C-1027 both decreases the amount of RPA available for DNA replication, through intranuclear redistribution, and inactivates RPA for DNA replication, but not for ssDNA binding.

Experimental Procedures

Chemicals and kinase assays. [α-^{32}P]dATP and [γ-^{32}P]ATP were obtained from Amersham Pharmacia Biotech. Adozelesin was generously supplied by Pharmacia Upjohn Co. (Kalamazoo, MI). Adozelesin stock solutions in dimethylacetamide (2 mg/ml) were diluted in dimethylsulfoxide prior to addition to 293 suspension cell culture. C-1027, a gift from Taiho Pharmaceuticals Co. Ltd, Saitama, Japan, was diluted in water and stored at –20°C.

Plasmids and proteins. The SV40 origin-containing plasmid pSV011 has been described previously (18). SV40 large T antigen (Tag) was purified from recombinant baculovirus infected High-Five™ insect cells (Invitrogen) using immunoaffinity chromatography (18). Bacterially expressed human Proliferating Cell Nuclear Antigen (PCNA) was purified as described (27). Recombinant baculoviruses encoding the five human Replication Factor C (RFC) subunits were a generous gift from Dr. Ellen Fanning,

Vanderbilt University. His-tagged human RFC complex was expressed from recombinant baculoviruses in High Five™ cells and purified by Ni-column chromatography and velocity gradient sedimentation as described (18). Topoisomerase I was purified from calf thymus as described (28). The DNA polymerase α and δ containing fraction IIA was purified from 293 cell extracts as described previously (27).

Cell cultures and antibodies. Human 293 cells were grown as suspension cultures in S-MEM (Life Technologies) containing 5% (v/v) calf serum. MO59J cells (ATCC) were maintained in DMEM/F12 supplemented with 0.05 mM non-essential amino acids and 10% fetal bovine serum. HeLa cells (ATCC) were maintained in DMEM with 10% fetal bovine serum. Monoclonal antibodies specific to the human RPA 70 kDa and 32 kDa subunits have been described previously (29).

Indirect immunofluorescent staining. MO59J and HeLa cells grown in two-well chamber slides were treated with 0, 0.1, 1 or 10 nM C-1027 at 37°C for the length of time specified in each Figure Legend. RPA was immunostained following the procedure of Swindle et al. (30) with minor modifications. Briefly, the cells were washed with 0.5% Triton X-100 in PBS, fixed with 3% paraformaldehyde and blocked with 50% normal goat serum in PBS. Monoclonal antibody against RPA32 was used as the primary antibody and incubated on the slide at 4°C for overnight. After extensive washing, fluorescein conjugated goat anti-mouse antibody (Vector Laboratory Inc.) was used as the secondary antibody and incubated at room temperature for 1 hr. After washing, the cells were examined using a Nikon Microphot microscope with Bio-Rad MRC-1024 confocal imaging system. Adobe Photoshop was used for image processing and printing.

Subcellular fractionation. The cellular fractionation was a slight modification of the protocol of Zou et al. (31). Briefly, $5x10^7$ 293 cells were harvested and washed with PBS

after treatment with 0 or 3 nM of C-1027 for 2 hr. The cyto/nucleosolic fraction was prepared by extracting protein from the harvested cells for 10 min on ice using a volume of buffer (0.5% Triton X-100 in CSK buffer - 10 mM PIPES pH 6.8; 300 mM sucrose; 100 mM NaCl; 3 mM $MgCl_2$; 1 mM EGTA) equal to the cell pellet volume. The residual nuclear structures were washed three times with PBS, and then incubated with 100 μg/ml of DNase I in CSK buffer at 37°C for 15 min, followed by the addition of ammonium sulfate to 0.25 M and further incubation at room temperature for another 10 min. The supernatant collected was designated the chromatin fraction. The remaining insoluble structures were washed with PBS and resuspended in SDS sample buffer with 20 mM Tris-HCl pH 7.5; 1 M 2-mercaptoethanol; 2% (w/v) sodium dodecyle sulfate and 10% glycerol.

Cell extract preparation and cell-free SV40 DNA replication assays. Whole cell extracts were prepared from 293 suspension cells after treating with DNA damaging agents at the indicated concentrations for 2 hr as described (18). In vitro SV40 DNA replication conditions, sample processing, and electrophoresis conditions were as described previously (18). Dried gels were exposed to Phosphorimager screens for various times, and DNA replication levels were evaluated using Phosphorimage quantification of the DNA replication products ranging from the replication intermediates (RI's) through the position of Form I DNA. Relative activity levels represent either pixel density/mm^2 x 1000 or percent synthesis (compared to the indicated control).

Purification of RPA. Suspension cultured 293 cells ($5x10^5$ cells/ml) were treated with 0 or 3 nM C-1027 for 2 hr at 37°C before they were harvested. The cell pellet was washed twice with phosphate buffered saline, resuspended in hypotonic buffer (20 mM

HEPES-K^+ pH 7.5; 0.5 mM EDTA; 1 mM $MgCl_2$; 0.1 mM PMSF; 1 mM DTT) and sonicated at medium power for 1 min. NaCl was then added to final 400 mM and the cell lysate was left on ice for 30 min. The soluble fraction was collected after centrifugation at 31,000 x g for 10 min, diluted with Buffer A (Tris-HCl pH 8.0 and 10 mM 2-mercaptoethanol) to a NaCl concentration at 100 mM and applied to a 20 ml Q-Sepharose column. The RPA was eluted from Q-Sepharose with Buffer A containing 500 mM NaCl. The eluent was applied to a 10 ml ssDNA cellulose column. After washing with Buffer A containing 750 mM NaCl, the RPA was eluted from the column with 20 mM Tris-HCl pH 8.0; 1.5 M NaCl; 10% glycerol, 40% ethylene glycol and 1 mM DTT. Protein fractions were further diluted with Buffer A, applied to a 100-µl Q-Sepharose column and eluted with 500 mM NaCl in Buffer A. The collected proteins were dialyzed against Buffer A containing 25 mM NaCl and 10% glycerol, aliquoted and stored at –80°C.

Western blot hybridization. Cell pellets from mock- or C-1027-treated 293 cells (5 x 10^6) were washed with cold PBS and lysed directly in SDS sample buffer. Extracts prepared from mock-, adozelesin- or C-1027-treated cells were mixed with equal volume of 2X SDS sample buffer (40 mM Tris-HCl pH 7.5; 4% SDS; 2 M 2-mercaptoethanol). Equal amounts of total protein were resolved by electrophoresis on 12.5% (w/v) SDS-polyacrylamide gels and transferred to Hybond-P membrane (Amersham Pharmacia Biotech) using NovaBlot (Amersham Pharmacia Biotech) as per the manufacturer's instructions. Membranes were probed with monoclonal antibodies against either largest subunit of RPA (RPA70) and/or RPA32. Peroxidase-conjugated goat anti-mouse IgG (Pierce) was used as the secondary antibody (at a 1:5,000 dilution) and detected using the Supersignal enhanced chemiluminescent reagent (Pierce) and exposure to X-ray film (Marsh).

Electrophoretic mobility shift assay (EMSA). Single-stranded DNA binding activity of RPA was evaluated using the electrophoretic mobility shift assay. Briefly, a 30 base oligodeoxynucleotide was 5'-end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase to a specific activity of 1-4x10^6 cpm/pmol. 1x10^4 cpm of labeled oligo was mixed with purified RPA (amounts indicated) in a 10 µl reaction mixture containing 30 mM Hepes-K^+ pH 7.5, 7 mM $MgCl_2$, 0.5 mM DTT and 0.1 mg/ml BSA for 15 min at room temperature. The reactions were separated on a 5% polyacrylamide gel (acrylamide:bisacrylamide ratio of 79:1) at 12 V/cm in 0.5 X TBE buffer.

Results

Extracts from C-1027-treated 293 cells are deficient in *in vitro* SV40 DNA replication. We have shown previously that treatment of human cells with C-1027 results in a decreased ability of extracts from treated cells to support SV40 DNA replication *in vitro* (26). This result was reexamined over a wider range of C-1027. Human 293 cells were treated with the indicated levels of C-1027 for 2 hours and extracts were prepared and tested for their ability to support SV40 DNA replication (Fig. 1). Compared to mock-treated cell extracts, extracts from C-1027-treated cells are clearly deficient in their ability to support SV40 DNA replication. The replication inhibition reached a plateau at between 5 and 10 nM C-1027, at which point DNA replication was decreased by over 95% (Fig. 1 A&B). To control for the possibility that the inhibition was due to C-1027 itself being carried into the replication reactions with the extracts from drug treated cells, C-1027 was added directly to mock-treated cell extracts prior to DNA replication. Addition of C-1027 to 20 nM final concentration had little negative effect on *in vitro*

DNA replication (Fig 1C). These results, together with our previously published data (26), suggest that C-1027 treatment-induced DNA replication arrest is mediated through cellular trans-acting factors, similar to results from previously published studies with different DNA damaging agents (8, 13-16, 18).

Reduced DNA replication activity could be due to either the loss of one or more essential DNA replication factors, or to the induction of a trans acting inhibitor of DNA replication. To differentiate between these two mechanisms, extracts from cells treated with C-1027 were mixed with mock-treated cell extracts to see whether the extracts from the drug treated cells had an inhibitory effect on DNA replication by the control cell extract. Addition of 10 to 30 μg of protein from cells treated with either 1 nM or 3 nM C-1027, to 40 μg of extract from untreated cells showed no trans inhibitory effect (Fig. 1D). This lack of a trans inhibitor is consistent with a loss of function of one or more essential DNA replication factors, and similar to results shown previously for the DNA alkylator, adozelesin (18). Potent trans inhibition was observed with extracts from cells treated with 10 nM C-1027 (Fig. 1D, filled squares). This trans inhibition is due to activation of DNA-PK[2] , and appears similar to inhibition by DNA-PK reported previously for camptothecin treatment (15). These results will be reported elsewhere.

Reduced SV40 DNA replication in extracts from cells treated with low doses of C-1027 is mediated through loss of RPA function. It has been shown previously for certain types of DNA damage that addition of exogenous Replication Protein A (RPA) is capable of rescuing the DNA replication activity of extracts from treated cells (16, 18). We therefore tested whether the inhibition of DNA replication upon treatment of cells with low doses of C-1027 is due to a loss of RPA function. While addition of up to 30 ng/μl of purified RPA had little effect on the DNA replication activity of mock-treated extracts, addition of RPA fully rescued the SV40 DNA replication activity in extracts

from cells treated with either 1 nM or 3 nM C-1027 (Fig. 2). This indicates that the primary mechanism of DNA replication inhibition following treatment of cells with low doses of C-1027 is through loss of RPA function. Since the addition of RPA protein alone into extracts from cells treated with low levels of C-1027 is able to fully rescue SV40 DNA replication activity, it suggests that all the other DNA replication factors remain both functional and at sufficient levels in these extracts. Addition of RPA could only partially rescue SV40 DNA replication activity in extracts from 10 nM C-1027-treated cells (Fig. 2). This is consistent with the cell extract mixing experiments above (Fig. 1D) which show that a trans inhibitor is induced upon treatment with high levels of C-1027.

Intra-nuclear redistribution of RPA. The loss of RPA function for DNA replication in extracts from cells treated with various DNA damaging agents could be caused either by reduced levels of RPA in cell extracts, or by RPA inactivation. Our previously published work suggested that RPA levels are decreased in extracts from C-1027 treated cells (26). RPA immunoblotting of extracts from cells treated with 1, 3 or 10 nM C-1027 show that these extracts contain only about 30% as much RPA as extracts from mock-treated cells (Fig. 3A, middle and lower panels, and data not shown). However, when mock- and C-1027-treated 293 cells were lysed directly in SDS sample buffer and analyzed by immunoblotting, total RPA levels remained the same (Fig. 3A, upper panel). These data indicate that reduced RPA protein levels in extracts from cells treated with C-1027 is due to a decrease in RPA extractability, not to lower levels of cellular RPA. These results, combined with the results from figure 2, suggest that the decrease in SV40 DNA replication activity in extracts from cells treated with 1 or 3 nM C-1027 is likely due to reduced RPA concentration in extracts from treated cells.

The fact that RPA levels remained constant in cells treated with C-1027 but decreased in cell extracts, implied that an appreciable fraction of RPA was being 'translocated' from the loosely associated nucleosolic fraction, which is extractable following hypotonic lysis, to a more tightly bound fraction. To address this possibility, mock- or 3 nM C-1027-treated 293 cells were separated into the following fractions: a combined cytosolic and nucleoplasmic fraction (cyto/nucleosolic), a chromatin bound fraction, and an insoluble fraction (see Experimental Procedures). These fractions were monitored for RPA levels using immunoblotting (Fig. 3C). For mock-treated cells, the majority of RPA (~70-80%) was found in the cyto/nucleosolic extract (lanes 8 and 9), while very little RPA was found in the chromatin bound or insoluble fractions (lanes 10 and 11), consistent with previously published results (32). However, cyto/nucleosolic extracts from cells treated with 3 nM C-1027 showed an appreciable reduction in levels of the RPA complex as evaluated by RPA70 levels (lanes 12 and 13). Following drug treatment, RPA levels in both the chromatin-bound (lane 14) and insoluble (lane 15) fractions increased dramatically. Interestingly, in C-1027-treated cells, the chromatin bound RPA population showed a much higher percentage of hyper-phosphorylated RPA32 than the other fractions.

The reported relationship between RPA and DNA-PK led us to also ask whether DNA-PK is required for the change in extractability of RPA following DNA damage. DNA-PK$^{(-/-)}$ (MO59J) cells were treated with either C-1027 or the DNA alkylating agent, adozelesin, for 2 hours. Immunoblotting for RPA showed that RPA32 is not hyper-phosphorylated in DNA-PK$^{(-/-)}$ cells treated with either DNA damaging agent (Fig. 3B, lanes 5 to 7). However, the appearance of extraction-resistant RPA was observed in DNA-PK$^{(-/-)}$ cells treated with C-1027 (Fig. 3B, compare lanes 5 and 7; and see additional results in Fig. 4 below). Extractable RPA levels were not changed by treatment with

adozelesin in either 293 cells (18), MO59J cells (Fig. 3B compare lanes 5 and 6), or the paired MO59K (DNA-PK$^{(+/+)}$) cells (data not shown). These data indicate that the change in RPA extractability is independent of RPA32 hyper-phosphorylation by DNA-PK, but is dependent on the type of DNA damage lesion.

It was recently demonstrated that treatment of cells with γ irradiation results in the appearance of RPA-containing foci within the nucleus of treated cells (33). We therefore investigated whether the extraction-resistant RPA in C-1027-treated cells also form foci. HeLa and DNA-PK$^{(-/-)}$ (MO59J) cells were treated with 0.1, 1 or 10 nM C-1027 for two hours. The cells were then washed with nonionic detergent before fixation, so the loosely bound nucleosolic RPA was removed (see Experimental Procedures). The stably bound RPA was visualized with a monoclonal antibody against RPA32 and a fluorescein conjugated secondary antibody. Less than half (~40%) of mock-treated cells showed a very low level of RPA staining (Fig 4A and 4E). Conversely, more than 90% of the C-1027-treated cells showed much stronger RPA staining, with the number of RPA foci increasing with drug dose (Fig. 4B-4D, 4F-4H). We also tested the time of treatment required for increased RPA staining. HeLa or MO59J cells were treated with 10 nM C-1027 for up to 2 hours. At 5 minutes of treatment, the RPA staining pattern was indistinguishable from mock-treated cells (Fig. 5A, 5A' and data not shown). However, by 10 minutes, the RPA signal was clearly stronger (Fig. 5B, 5B'). The intensity increased continuously up to 40 minutes at which point there was no further increase in intensity (Fig. 5B-5E). Since the time required for induction of DNA damage by C-1027 treatment is 5 to 10 minutes (34), RPA focus formation must be a very early DNA damage response event. The fact that the same C-1027-induced RPA staining pattern is seen in both DNA-PK$^{(-/-)}$ cells and in HeLa cells (Figs. 4 and 5) further supports the results from figure 3, indicating that the induction of

extraction-resistant RPA is independent of DNA-PK activity and RPA32 hyper-phosphorylation.

RPA inactivation. The fact that RPA levels remain constant in cell extracts following adozelesin treatment (Fig. 3B), while these extracts are nonetheless deficient in RPA DNA replication activity (18), led us to hypothesize that the RPA in DNA damaged cells may be inactivated for DNA replication. Previous studies with UV-treated cells suggested a similar possibility (17, 35). To test this hypothesis directly, RPA was purified from 293 cells treated with 0 or 3 nM C-1027 using Q Sepharose and ssDNA affinity chromatography as described in the Methods. The various fractions prepared during the purification were then subjected to RPA immunoblotting. As shown in Fig. 6A (upper panel), about 30% of the total RPA32 from C-1027-treated cells is hyper-phosphorylated (lane 1). Interestingly, the flow-through of the ssDNA cellulose column contains predominantly un-phosphorylated RPA32, but very little RPA70 or hyper-phosphorylated RPA32 (lane 3). Conversely, most of the RPA70 and the hyper-phosphorylated RPA32 are present in the eluent from the ssDNA cellulose column. The percentage of RPA32 in this fraction that is hyper-phosphorylated is approximately 70% (lane 4 and data not shown). RPA from mock-treated 293 cells show a similar percentage of RPA32 in the flow-through of ssDNA cellulose, again with very little RPA70, and similar levels of RPA complex in the ssDNA cellulose column eluent (Fig. 6A, lower panel). Based on these results, we conclude that (1) there is about 30% of the total RPA32 in 293 cell extracts that is not associated with RPA70, consistent with previous reports (32); (2) only the RPA32 in the heterotrimeric form of RPA appears to be hyper-phosphorylated following DNA damage; (3) the percentage of hyper-phosphorylated RPA32 in the heterotrimeric form following DNA damage is much higher than the previously reported observations of 25 to 30%; and (4) the hyper-

phosphorylated RPA heterotrimer is stable in 750 mM NaCl (which is used during the wash of the ssDNA cellulose column) to 1.5 M NaCl (used to elute the column). In addition, these ssDNA cellulose eluted RPA trimeric complexes can be immunoprecipitated with either RPA70 or RPA32 monoclonal antibodies (data not shown). This latter result appears to conflict with previously published data showing that hyper-phosphorylated RPA32 dissociates from RPA70 in 400 mM NaCl (22).

The RPA purified from C-1027 treated 293 cells was compared to RPA purified similarly from mock-treated cells in ssDNA binding assays and partially reconstituted SV40 DNA replication assays. The ssDNA binding activities of the two RPA preparations were very similar (Fig. 6B). However, when tested in a partially reconstituted SV40 DNA replication assay dependent on the addition of RPA, the RPA purified from C-1027-treated cells supported SV40 DNA replication to levels only about 30% of those supported by equivalent levels of RPA purified from mock-treated cells (Fig 7). Addition of higher levels of RPA resulted in little additional synthesis (Fig. 7 and data not shown). This is the first direct evidence showing that RPA from cells treated with a genotoxic agent is inactivated for DNA replication. RPA purified from adozelesin-treated cells showed similar results (data not shown).

Discussion

In this report we have examined the mechanisms by which treatment of cells with the DNA damaging agent C-1027 results in inhibition of DNA replication. Analysis of data presented here, in conjunction with previously published work, has demonstrated that there are multiple mechanisms for inhibiting DNA replication in response to DNA damage. These mechanisms include: transfer of RPA from a loosely

associated nucleosolic fraction to tightly associated foci, inactivation of RPA for DNA replication, and induction of trans-acting inhibitors. Herein we have demonstrated that RPA is both transferred to an extraction-resistant fraction and inactivated in C-1027 treated cells (Figs. 3 to 6). These results suggest a unique role for RPA in DNA damage-induced DNA replication arrest.

RPA is a relatively abundant nuclear protein in eukaryotic cells and is required for DNA replication, repair and recombination. Overall RPA protein levels do not change during different phases of the cell cycle or upon DNA damage (Fig. 3) (5). Indirect immunofluorescent staining of RPA in cells fixed prior to any extraction shows signal throughout the nucleus (36). Washing with nonionic detergent prior to fixation removes most of the RPA, leaving small RPA-stained sites only in S phase cell nuclei. These sites colocalize with other DNA replication factors and sites of newly synthesized DNA referred to as DNA replication foci (36-40). Detection of similar RPA foci on damaged DNA in γ-irradiated cells has also been reported (33). We have shown that treatment of cells with C-1027 results in a rapid and dramatic change in the RPA distribution (Figs. 4 & 5). More than 90% of the C-1027-treated cells show the appearance of nonionic detergent-stable RPA foci within 10 minutes of treatment. This is reflected by a decrease in extractability of a large portion of the cellular RPA. Unlike the majority of RPA in untreated cells, this RPA population is highly resistant to extraction by both salt and nonionic detergent. Treatments of cells with genotoxic agents have been shown to both decrease the extractability of other nuclear proteins, such as hRad9 (41) and proliferating cell nuclear antigen (PCNA)[2], and to localize them to intranuclear foci. When cells are treated with agents that lead to dsDNA breaks, Mre11, 53BP1 and histone H2AX all form nuclear foci within a short time after induction of damage (42-44). The number of RPA foci induced by treatment with C-1027 appears

to be much higher than the number of Mre11 and H2AX foci reported to be induced by other DNA damaging treatments; however, this could be due to the different DNA damaging agents used. Our preliminary results show that C-1027-treated, nonionic detergent-washed cells show much stronger levels of RPA signal than Mre11, making possible colocalization difficult to establish. One potential reason is that unlike other DNA damage marker proteins, such as the Mre11/Rad50/NBS1 complex, 53BP1, and histone H2AX, which predominantly bind to dsDNA breaks, RPA also binds to ssDNA and to damaged DNA without strand breaks (e.g. - RPA associates with cis-platin damaged DNA (45, 46)). Therefore, one would expect that only a subset of DNA damage-induced RPA foci would co-localize with these other markers. The relationship between extraction-resistant RPA, DNA damage-induced RPA foci, and other DNA damage markers is under continuing investigation. Based on the available data, we hypothesize that there is a large pool of RPA in normal cell nuclei that is ready to rapidly assemble into either tightly associated DNA replication complexes (in S phase cells) or into various types of DNA repair or recombination complexes at sites of damaged DNA.

Hyper-phosphorylation of RPA32 appears not to contribute to the increased extraction-resistance or focalization of RPA. This is supported by several lines of evidence. First, RPA was found to become equally extraction-resistant in DNA-PK$^{(-/-)}$ (MO59J) cells treated with C-1027 with no detectable RPA32 at the hyper-phosphorylated position (Fig. 3B). Second, C-1027-treatment of MO59J cells induces RPA foci (Fig. 4). Third, treating cells with very low levels of C-1027 (between 0.1 and 0.5 nM) results in the appearance of RPA foci (Fig. 4). However, these levels of C-1027 do not induce hyper-phosphorylation of RPA32 as evaluated by western blot analysis (26, and data not shown). Finally, adozelesin treatment of 293 cells, which induces

RPA32 hyper-phosphorylation (18), does not induce appreciable levels of focalization or extraction-resistance of RPA (Fig. 3B).

Results from prior studies have suggested that treatment of cells with certain DNA damaging agents results in a loss of RPA activity, as the addition of RPA and RPA alone can rescue SV40 DNA replication in extracts from treated cells (16, 18, 35). While this has been proposed to be due to decreased RPA levels in extracts from treated cells (15), in this report we demonstrate the first direct evidence that RPA is also inactivated as a DNA replication factor in cells treated with certain DNA damaging agents. Importantly, the levels and kinetics of drug treatment that result in the inactivation of RPA for *in vitro* DNA replication are the same as that result in inhibition of DNA replication in cell culture (9, 10, 12, 18). These results argue strongly that RPA inactivation for DNA replication reflects an authentic cellular DNA damage response. The inactivation of RPA for DNA replication also appears to be a highly specific response, since RPA purified from drug-treated cells appears to show normal ssDNA binding. We anticipate that this will be reflected in the ability of this modified RPA to support other critical RPA functions in repair and recombination, biochemical processes that are likely to be playing critical roles in the repair of the very DNA damage that triggers the inactivation of the replication function of RPA. Studies on the function of modified RPA in repair and recombination are currently in progress.

Additional preliminary results indicate that dephosphorylation of this inactive RPA can rescue its ability to support SV40 DNA replication *in vitro*[2]. While RPA has been shown to be phosphorylated by a variety of kinases (for reviews see 5, 6), it has been shown that *in vitro* phosphorylation of RPA by DNA-PK and/or cyclin-cdk2 does not inactivate RPA for DNA replication (47-50). While we have shown that DNA damage-triggered *in vivo* phosphorylation can inactivate RPA for DNA replication, our

results are nonetheless not inconsistent with these previous studies. We presume that the inactivating phosphorylation event must be carried out by a different kinase. In further support of this, we have found that like other cells, extracts from DNA-PK$^{(-/-)}$ (MO59J) cells treated with either C-1027 or adozelesin are also inhibited in their ability to support DNA replication *in vitro*. This inhibition can also be overcome by the addition of RPA to these extracts, indicating that even in the absence of DNA-PK function, RPA is being inactivated for DNA replication[2]. Based on the previous studies and our own findings, we conclude that the inactivation of RPA must occur through a different kinase than DNA-PK or cdk. It has been shown that the large subunit of RPA is phosphorylated following DNA damage in *S. cerevisiae* (51). The identity of the kinase responsible for RPA70 phosphorylation is still unclear and the effect of this modification on RPA's function in DNA replication and repair requires further study.

The role of the DNA damage-dependent hyper-phosphorylation of RPA32 by DNA-PK remains unknown. Numerous studies have shown a lack of effect of DNA-PK phosphorylation on the ability of RPA to support DNA replication. Our results are consistent with these previous studies. Results presented in this study further indicate that DNA-PK does not appear to be required for DNA damage-dependent changes in RPA extractability and RPA focalization. One possibility is that hyper-phosphorylation of RPA32 by DNA-PK may be involved more directly in the ability of RPA to support either DNA repair or recombination. At least some of the cellular RPA is normally bound by DNA-PK, and it is both released and hyper-phosphorylated by DNA-PK following DNA damage (24). This is particularly intriguing in light of the essential role that DNA-PK plays in cellular DNA recombination, and may suggest a role for RPA hyper-phosphorylation in DNA recombination.

The fact that we have identified multiple mechanisms for DNA replication inhibition induced by different DNA damaging agents argues strongly that there exist multiple DNA-damage inducible S phase checkpoint mechanisms. This is not altogether surprising, since it is clear that different types of DNA damage must be recognized and repaired by different DNA repair systems, and these lesions are therefore likely to be identified by different DNA damage detector molecules. These same detectors would presumably trigger the cellular DNA damage response pathways, including those S phase checkpoint pathways that need to act immediately to halt both DNA replication initiation and fork progression. While one could have predicted that the various DNA damage detectors might feed into the same pathway for inhibiting DNA replication, the importance of preventing DNA synthesis through damaged DNA is a strong rationale for having multiple DNA replication arrest mechanisms. The results presented herein provide support for the existence of multiple mechanisms triggered by different types of DNA damage.

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Footnotes

1. The abbreviations used are: RPA, replication protein A; ssDNA, single-strand DNA; dsDNA, double-strand DNA; SV40, simian virus 40; DNA-PK, DNA-dependent protein kinase.

2. J. S. Liu, S. R. Kuo and T. Melendy unpublished results.

Figure Legends

Fig. 1. Treatment of cells with C-1027 inhibits the ability of extracts to support SV40 DNA replication. A. 40 to 70 μg of extract from cells treated with 0, 1, 3 or 10 nM C-1027 were used in SV40 DNA replication assays. The migration of supercoiled Form I DNA, open circular Form II DNA and theta form replication intermediates (R.I.) are indicated to the right of the panel. The quantitated results from A are shown in B. C. C-1027 was added to SV40 DNA replication assays with mock-treated cell extracts to final concentrations of 0 to 20 nM. The level of synthesis without additional C-1027 was defined as 100%. D. 0 to 30 μg of extract from cells treated with 0 nM (open square), 1 nM (triangle), 3 nM (circle) or 10 nM (filled square) C-1027 were mixed with 40 μg of mock-treated cell extract and tested in SV40 DNA replication assays.

Fig. 2. Effect of exogenous purified RPA on SV40 DNA replication with extracts from cells treated with C-1027. Zero to 30 ng/μl of RPA was added to SV40 DNA replication reactions with extracts from cells treated with 0 to 10 nM C-1027. Activity from mock-treated extracts with no additional RPA was defined as 100%.

Fig. 3. RPA protein levels in drug-treated cells and different subcellular fractions. A. $5x10^7$ suspension cultured 293 cells were treated with 0 to 10 nM of C-1027 for 2 hr. $5x10^5$ cells were lysed directly into SDS sample buffer. The remaining cells were used to prepare cell extracts. Total protein from the whole cells (top panel) or cell extracts (middle and lower panel) were resolved by electrophoresis on 12.5% (w/v) SDS-polyacrylamide gels, transferred to Hybond-P membrane and probed with antibodies against RPA70 and RPA32. B. Extracts from mock-, adozelesin- and C-1027-treated MO59J cells are analyzed as described above. C. Subcellular fractions from mock- and C-1027-treated 293 cells were analyzed via immunoblotting with RPA70 and RPA32 specific antibodies. The amount of each fraction loaded onto the gel represents the

following percentage of the total volume of that fraction: Total (lanes 8 and 12), 0.8%; Cyto/nucleosolic (lanes 9 and 13), 1%; Chromatin (lanes 10 and 14), 3%; Insoluble (lanes 11 and 15), 3%.

Fig. 4. Indirect immunostaining of RPA in HeLa and MO59J cells treated with increasing doses of C-1027. Monolayer cultured HeLa (A-D) or MO59J (E-H) cells were treated with 0 nM (A and E), 0.1 nM (B and F), 1 nM (C and G) or 10 nM (D and H) C-1027 for 2 hrs. The cells were then washed with 0.5% Triton X-100, fixed with paraformaldehyde and stained for stably bound RPA with monoclonal antibody against RPA32 and fluorescein labeled goat anti-mouse antibody as described in the Experimental Procedures. Similar exposures were used for all frames.

Fig. 5. Indirect immunostaining of RPA in MO59J cells treated with C-1027 for different lengths of time. MO59J cells were treated with 10 nM C-1027 for 5 min to 2 hr, washed with nonionic detergent, and stained for stably bound RPA as described in the Experimental Procedures. The time of drug treatment is indicated in each frame. Similar exposures were used for frames A through E. Frames A′, B′ and C′ are identical to A, B and C, but represent a longer exposure.

Fig. 6. Purification and ssDNA binding of RPA from C-1027-treated 293 cells. A. Fractions from each step of RPA purification as described in Experimental Procedures were analyzed via immunoblotting with RPA70 and RPA32 specific antibodies. RPA70 and RPA32 from C-1027-treated cells (upper panels) and mock-treated cells (lower panels) are labeled on the right. B. Electrophoretic mobility shift assays of a 30-nucleotide oligo with 0 to 30 ng of RPA purified from mock-treated (lanes 2 to 4) or C-1027-treated (lanes 5 to 7) cells were performed as described in the Experimental Procedures.

Fig. 7. Functional assays of RPA in partially reconstituted SV40 DNA replication reactions. A. 7.5, 15 or 30 ng/μl of RPA purified from mock- or C-1027-treated 293 cells were tested in partially purified, RPA-dependent SV40 DNA replication assays. Assays were carried out as described in the Experimental Procedures. To generate an RPA-dependent assay, optimized levels of purified PCNA, RFC, topoisomerases, and fraction IIA (which supplies DNA polymerases alpha and delta) were used in place of the crude cell extract. Migration of the replication intermediates (R.I.) and Form II DNA are indicated on the left. B. Results from panel A were quantified using PhosphorImager analysis as described in the Experimental Procedures.

Fig 1 - Liu et al.

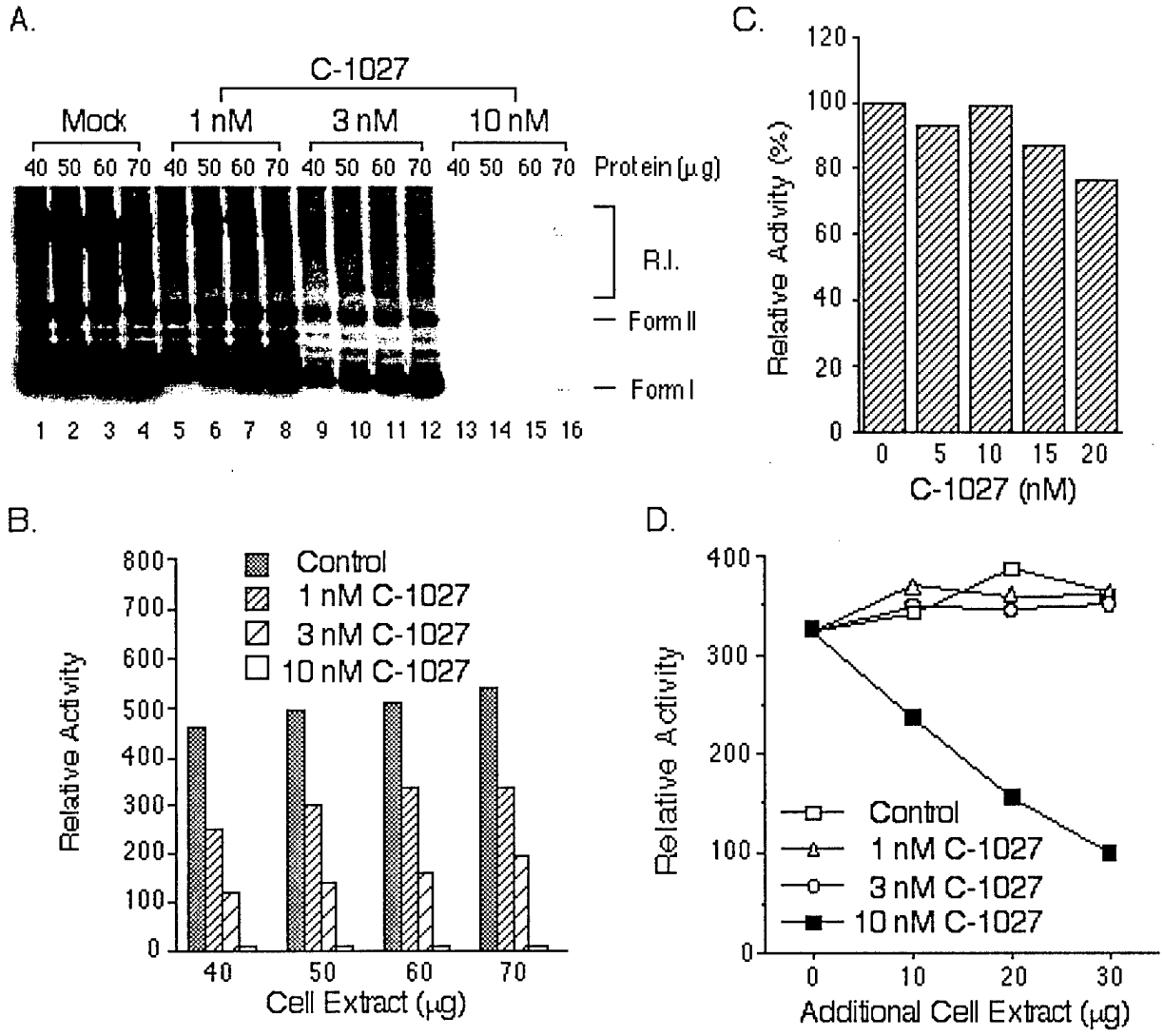


Fig 2 - Liu et al.

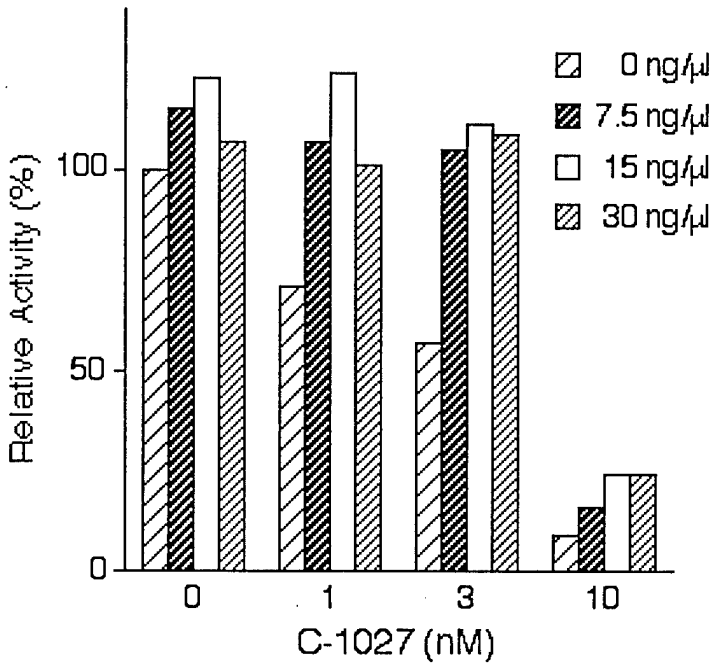
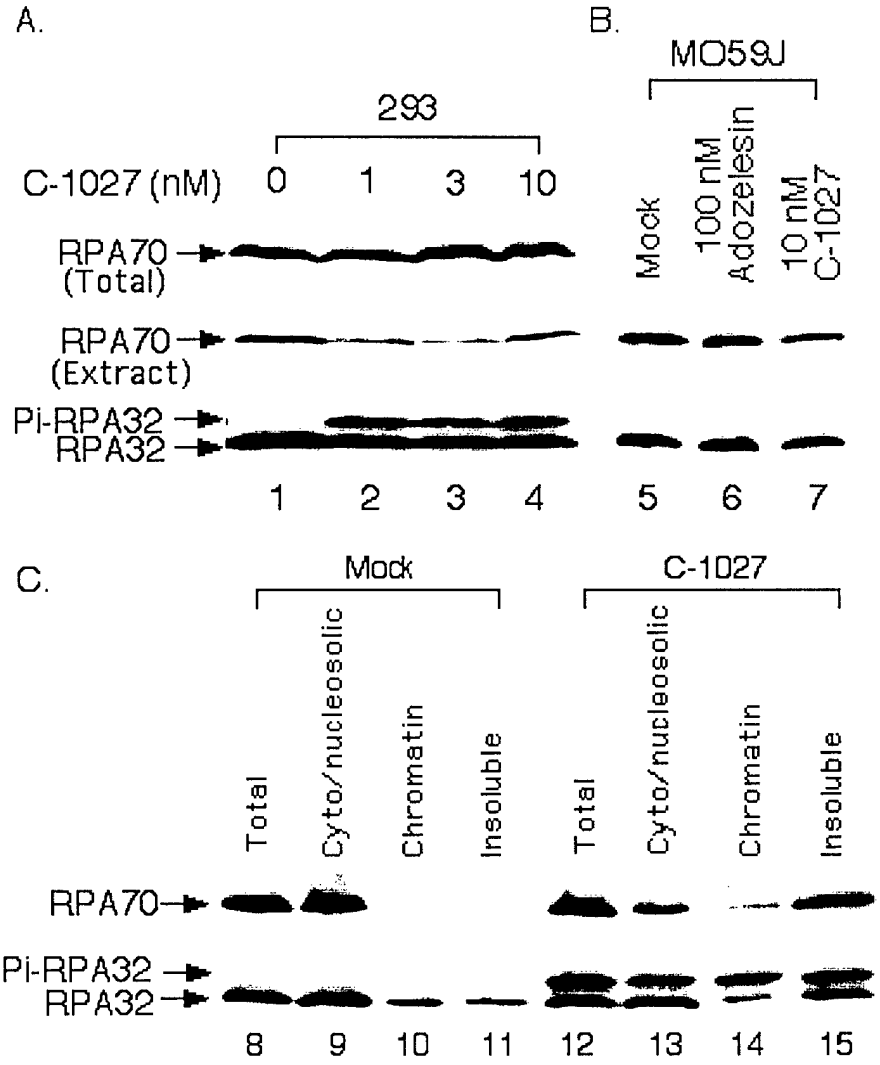


Fig 3 - Liu et al.



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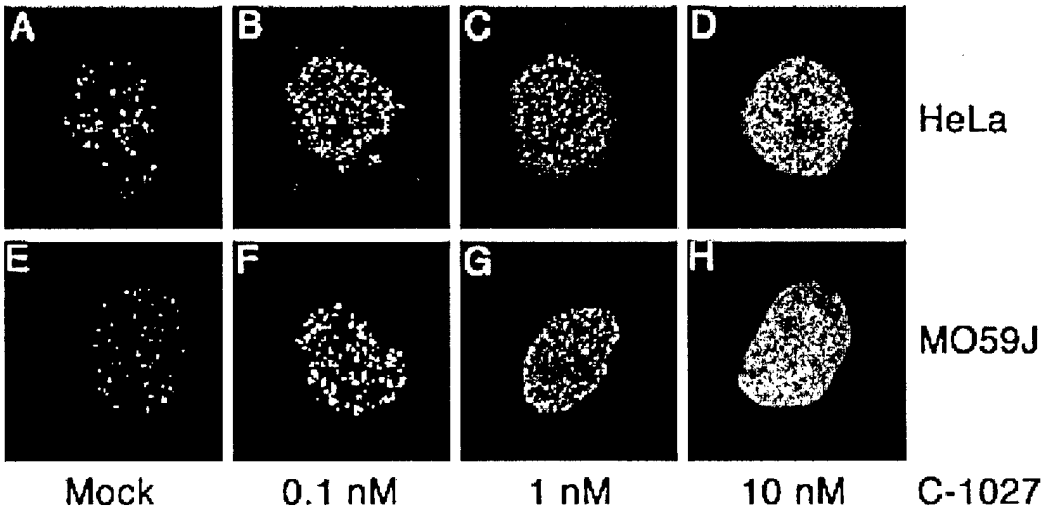

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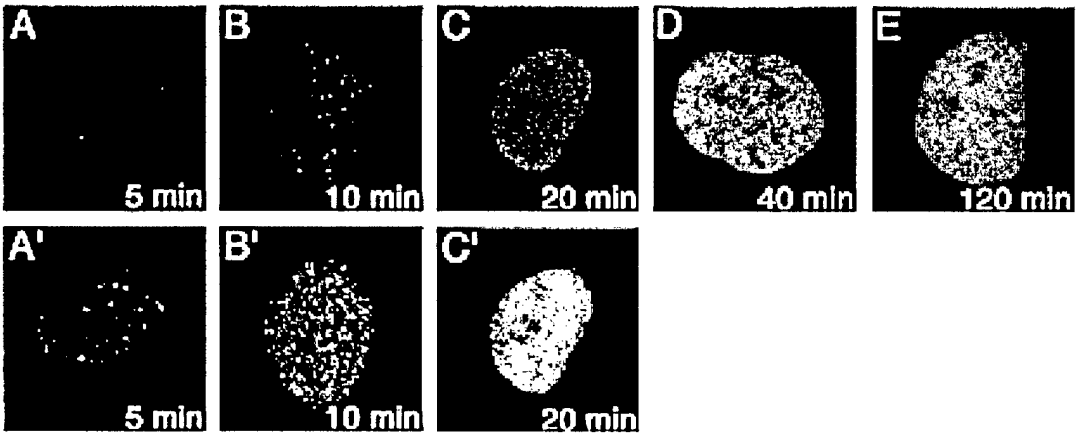

Figure 6 - Liu et al.

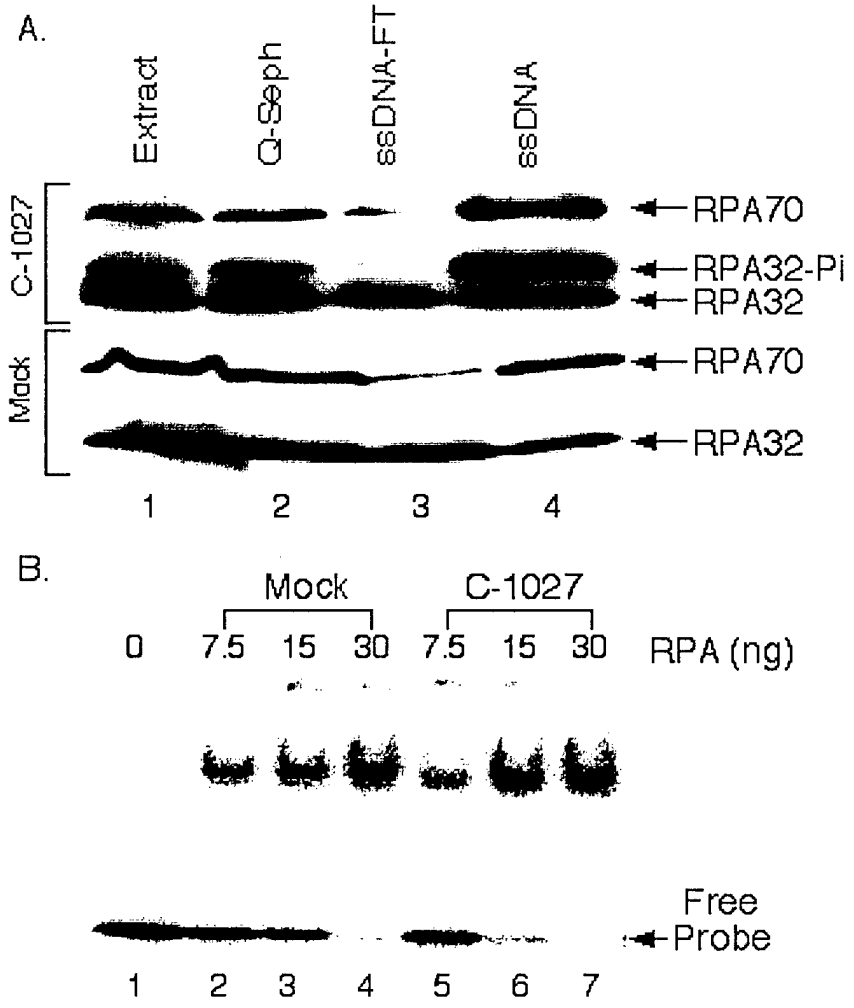

Fig 7 - Liu et al.